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- (71) Applicants: **THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ILLINOIS** [US/US]; 352 Administration Building, 506 South Wright Street, Urbana, IL 61801 (US). **ADVANCED LIFE SCIENCES INC.** [US/US]; 12305 South New Avenue, Lemont, IL 60439 (US).
- (72) Inventors: **PEZZUTO, John, M.**; 846 Bonnie Brae Place, River Forest, IL 60305 (US). **KOSMEDER, Jerome, W., II**; 215B Washington Boulevard, #2, Oak Park, IL 60302 (US). **XU, Ze-Qi**; 6609 Chick Evans Lane, Woodridge, IL 60517 (US). **ZHOU, Nian, En**; 2204 Mercer Court, Naperville, IL 60565 (US).
- (74) Agent: **NAPOLI, James, J.**; Marshall, Gerstein & Borun, 6300 Sears Tower, 233 South Wacker Drive, Chicago, IL 60606 (US).
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WO 02/16395 A1

(54) Title: PRODRUGS OF BETULINIC ACID DERIVATIVES FOR THE TREATMENT OF CANCER AND HIV

(57) Abstract: A composition and method of preventing or inhibiting tumor growth and, more particularly, of treating a malignant tumor, using prodrugs of plant-derived compounds and derivatives is disclosed. In the method, a composition containing betulinic acid or a betulinic acid derivative is administered in a prodrug form to release betulinic acid or a betulinic acid derivative *in vivo* at the tumor site.

## PRODRUGS OF BETULINIC ACID DERIVATIVES FOR THE TREATMENT OF CANCER AND HIV

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of  
5 U.S. provisional patent application No. 60/226,536,  
filed August 18, 2000.

FIELD OF THE INVENTION

The present invention relates to compositions and methods of inhibiting tumors and, more  
10 particularly, of treating a malignant tumor, like a  
melanoma, using plant-derived compounds and derivatives thereof, and in particular, prodrugs of betulinic acid and betulinic acid derivatives.

BACKGROUND OF THE INVENTION

15 Over the past four decades the incidence  
of melanoma has been increasing at a higher rate  
than any other type of cancer. It is now theorized  
that one in ninety American Caucasians will develop  
malignant melanoma in their lifetime. While an in-  
20 creasing proportion of melanomas are diagnosed  
sufficiently early to respond to surgical treatment  
and achieve a greater than 90% ten-year survival  
rate, it is estimated that nearly 7,000 individuals  
suffering from metastatic melanoma will die in the  
25 United States each year.

For patients afflicted with a metastatic  
melanoma not amenable to surgical extirpation,  
treatment options are limited. 5-(3,3-Dimethyl-1-  
triazenyl)-1-H-imidazole-4-carboxamide (dacarbazine,

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DTIC) is the most efficacious single chemotherapeutic agent for melanoma having an overall response rate of 24%. But the duration of response to DTIC is generally quite poor. Combination therapy with  
5 other synthetic and recombinant agents, including N,N'-bis(2-chloroethyl)-N-nitrosurea (carmustine, BCNU), cisplatin, tamoxifen, interferon-alpha (INF- $\alpha$ ), and interleukin-2 (IL-2), has a higher response rate (e.g., 30-50%) in some trials, but a durable  
10 complete response rate is uncommon and toxicity is increased. Sequential chemotherapy also has promise, but current treatment options for individuals suffering from metastatic melanoma are unsatisfactory.

15 Various drugs derived from natural products, such as adriamycin (doxorubicin), bleomycin, etoposide, and vincristine, and their derivatives, have been tested for efficacy against melanoma either as single agents or in combination therapy.  
20 However, similar to the synthetic and recombinant compounds, these compounds exhibit low response rates, transient complete responses, and high toxicities.

Nonetheless, as demonstrated by known and  
25 presently used cancer chemotherapeutic agents, plant-derived natural products are a proven source of effective drugs. Two such useful natural product drugs are paclitaxel (taxol) and camptothecin.  
Paclitaxel, originally derived from the bark of the  
30 Pacific yew tree *Taxus brevifolia* Nutt. (Taxaceae), currently is used for the treatment of refractory or residual ovarian cancer. More recently, clinical trials have investigated the possible role of paclitaxel in the treatment of metastatic melanoma.

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As a single agent, taxol displays activity comparable to cisplatin and IL-2. Taxol functions by a unique mode of action, and promotes the polymerization of tubulin. Thus, the antitumor response  
5 mediated by taxol is due to its antimitotic activity.

The second drug of prominence, camptothecin, was isolated from the stem bark of a Chinese tree, *Camptotheca acuminata* Decaisne (Nyssaceae).  
10 Camptothecin also functions by a novel mechanism of action, i.e., the inhibition of topoisomerase I. Phase II trials of a water-soluble camptothecin pro-drug analog, irinotican (CPT-11), have been completed in Japan against a variety of tumors with  
15 response rates ranging from 0% (lymphoma) to 50% (small cell lung). Topotecan, another water-soluble camptothecin analog, currently is undergoing Phase II clinical trials in the United States.

In addition, studies have shown that betulinic acid, and betulinic acid derivatives, can inhibit other types of cancer cells, such as neuroblastoma, in addition to melanoma. For example, Das  
20 Gupta et al. U.S. Patent No. 5,658,947 discloses that betulinic acid is useful for the selective control or treatment of human melanoma, and Pezzuto  
25 et al. U.S. Patent No. 5,962,527 discloses the selective activity of derivatives of betulinic acid against melanoma cells.

However, a disadvantage associated with  
30 the use of betulinic acid or a betulinic acid derivative in the treatment of a cancer is the problem encountered in formulating these active drugs and in providing suitable dosage forms for the treatment of various cancers. The present application is direc-

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ted to overcoming this disadvantage and providing useful prodrugs of betulinic acid and derivatives thereof that are easy to formulate into a variety of dosage forms and that release betulinic acid or the  
5 derivative thereof *in vivo*.

#### SUMMARY OF THE INVENTION

The present invention is directed to a method and composition for preventing or inhibiting tumor growth. The active compound is a prodrug of  
10 betulinic acid or a betulinic acid derivative which generates betulinic acid or a derivative thereof *in vivo*. Betulinic acid is a natural product obtained by a method comprising the steps of preparing an extract from the stem bark of *Ziziphus mauritiana*  
15 and isolating the betulinic acid. Alternatively, betulin can be isolated from the extract, and betulinic acid then is prepared from betulin by a series of synthetic steps.

Betulinic acid can be isolated from the  
20 extract by mediating a selective cytotoxic profile against human melanoma in a subject panel of human cancer cell lines, conducting a bioassay-directed fractionation based on the profile of biological activity using cultured human melanoma cells (MEL-2)  
25 as the monitor, and obtaining betulinic acid therefrom as the active compound. The resulting betulinic acid can be used to prevent or inhibit tumor growth, or can be converted to a derivative to prevent or inhibit tumor growth.

30 The physiochemical properties of betulinic acid, e.g., a high melting point and limited solubility in hydrophilic and hydrophobic solvents, make

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it difficult to produce betulinic acid-containing pharmacological formulations. The present invention is directed to providing betulinic acid or a betulinic acid derivative in a form that is easy to  
5 formulate and wherein endogenous enzymes can release the active betulinic acid or derivative *in vivo*.

Therefore, an important aspect of the present invention is to provide a method and composition for preventing or inhibiting tumor growth  
10 and, particularly, for preventing or inhibiting the growth of a malignant tumor using a natural product-derived compound, or a derivative thereof, in an easy-to-formulate form.

Another aspect of the present invention is  
15 to improve the bioavailability of betulinic acid and betulinic acid derivatives in an individual by administering a therapeutically effective amount of a prodrug of betulinic acid or betulinic acid derivative to an individual in need thereof.

Another aspect of the present invention is  
20 to provide a treatment method utilizing a prodrug of betulinic acid or derivative thereof to prevent the growth or spread of cancer cells, wherein betulinic acid or a derivative thereof is administered to an  
25 individual in need thereof in a manner consistent with the treatment of a cancer sensitive to betulinic acid or a derivative thereof, e.g., in a topical preparation for the prevention, inhibition, or treatment of melanoma, or intravenously or intra-  
30 peritoneally for other forms of cancer.

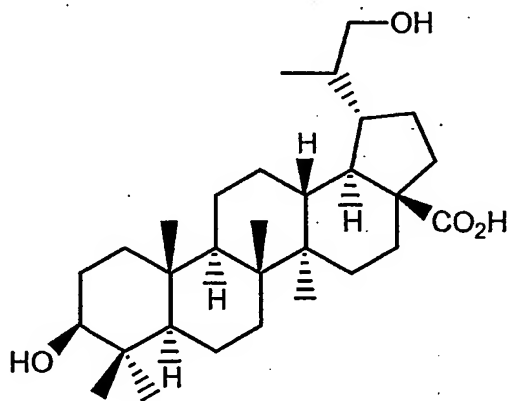
Yet another aspect of the present invention is to overcome the problem of high mammalian toxicity associated with synthetic anticancer agents by using a natural product-derived compound, e.g., a

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prodrug of betulinic acid or a betulinic acid derivative.

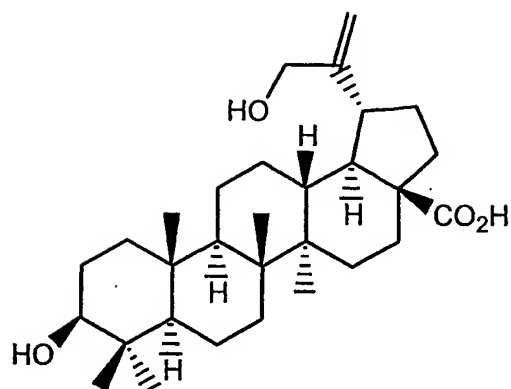
Yet another aspect of the present invention is to provide a composition and method of treating various forms of cancer with a naturally occurring product, or a derivative thereof. In particular, the present invention is directed to inhibiting malignant tumor growth associated with melanoma, neuroblastoma, breast cancer, lung cancer, fibrosarcoma, colon cancer, oral epidermoid carcinoma, epidermoid carcinoma, prostate cancer, hormone-dependent breast cancer, and glioma.

In particular, an aspect of the present invention is to provide a composition for treating tumor growth comprising:



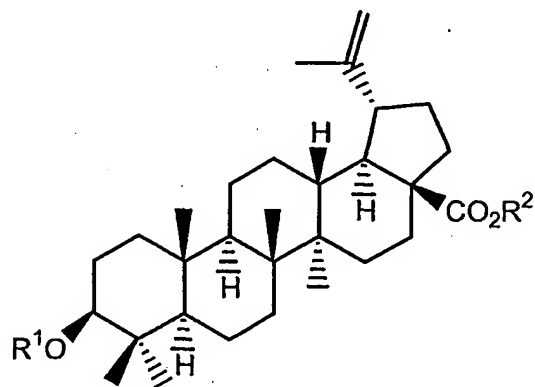
or

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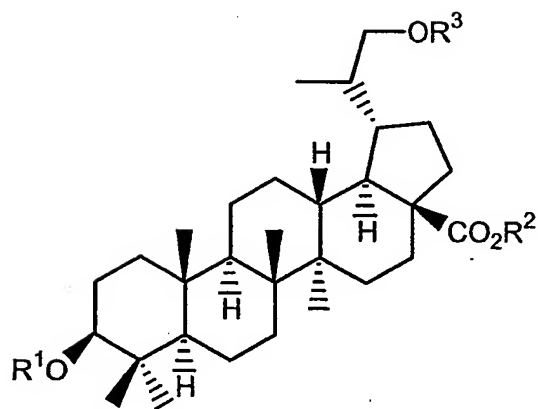
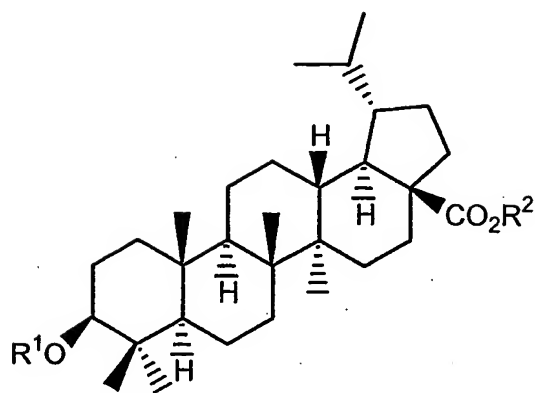
and (b) an optional carrier.

Another aspect of the present invention is  
to provide a composition for treating tumor growth  
5 comprising:



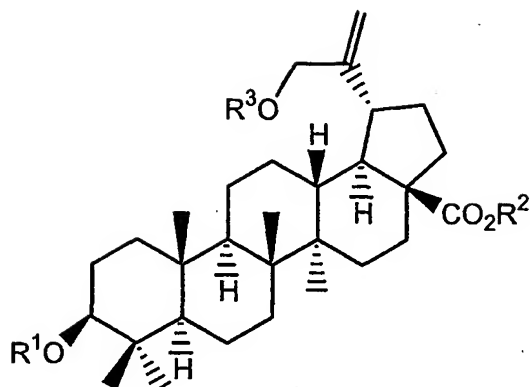


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, and

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wherein  $R^1$  and  $R^3$ , independently, are selected from the group consisting of hydrogen,  $\text{CO}(\text{C}_1\text{-C}_6\text{alkyl})\text{NR}^4\text{R}^5$ ,  $\text{CO}(\text{C}_{1-3}\text{alkyl})\text{CO}_2\text{R}^4$ ,  $\text{COCH}(\text{C}_6\text{H}_5)\text{NR}^4\text{R}^5$ ,  $\text{CO}(\text{C}_1\text{-C}_6\text{alkyl})$ ,  $\text{CO}(\text{C}_1\text{-C}_6\text{alkyl})\text{CO}_2\text{R}^4$ ,  $\text{CO}(\text{C}_{1-6}\text{alkyl})\text{O}$ -  
 5  $(\text{CH}_2\text{CH}_2\text{O})_n\text{C}_{1-3}\text{alkyl}$ ,  $\text{CH}_2\text{OCO}_2\text{C}_{1-6}\text{alkyl}$ ,  $\text{CH}_2\text{OCOC}_{1-6}\text{alkyl}$ ,  $\text{PO}(\text{OH})_2$ , and  $\text{SO}_3\text{H}$ ,

$R^2$  is selected from the group consisting of hydrogen,  $\text{C}_1\text{-C}_6\text{alkyl}$ ,  $\text{CH}_2\text{C}_6\text{H}_5$ ,  $\text{C}_1\text{-C}_6\text{alkylNR}^4\text{R}^5$ ,  $\text{CH}_2\text{OCOC}_{1-6}\text{alkyl}$ ,  $\text{PO}(\text{OH})_2$ ,  $\text{SO}_3\text{H}$ ,  $\text{CH}(\text{C}_6\text{H}_5)\text{NR}^4\text{R}^5$ ,  $(\text{C}_1\text{-C}_6\text{alkyl})\text{CO}_2\text{R}^4$ ,  
 10 and  $(\text{C}_1\text{-C}_6\text{alkyl})\text{O}(\text{CH}_2\text{CH}_2\text{O})_n\text{C}_{1-3}\text{alkyl}$ ,

$R^4$  and  $R^5$ , independently, are selected from the group consisting of hydrogen,  $\text{C}_1\text{-C}_6\text{alkyl}$ ,  $\text{CO}(\text{C}_1\text{-C}_6\text{alkyl})$ , and aryl, or  $R^4$  and  $R^5$  can be taken together to form a 5 to 7 membered ring,

15 and  $n$  is 1 to 10;

and pharmaceutically acceptable salts thereof,

and (b) an optional carrier.

Yet another aspect of the present invention is to provide a method of treating cancer  
 20 sensitive to betulinic acid or a derivative thereof comprising administering to an individual in need

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thereof a therapeutically effective amount of a pro-drug of betulinic acid or a derivative of betulinic acid. In particular, wherein the cancer is selected from the group consisting of a melanoma, a squamous tumor, a breast cancer, a colon cancer, a sarcoma, a human oral epidermal carcinoma, a hormone-dependent breast cancer, a prostate cancer, a lung cancer, a glioma, a melanoma, and a neuroblastoma.

These and other aspects of the present invention will become apparent from the following description of the invention, which are intended to limit neither the spirit nor scope of the invention but are only offered as illustrations of the preferred embodiments of the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Betulinic acid,  $3\beta$ -hydroxy-lup-20(29)-ene-28-oic acid, is a natural, pentacyclic triterpene product isolated from several genus of higher plants. Betulinic acid has demonstrated remarkable selective antitumor activity against human melanoma (E. Pisha et al., *Nature Medicine*, 1, pp. 1046-1051 (1995)) and anti-HIV activity (T. Fujioka et al., *J. Nat. Prod.*, 57, pp. 243-247 (1994)).

Through a bioassay-directed fractionation of the stem bark of *Ziziphus mauritiana* Lam. (Rhamnaceae), betulinic acid was isolated as an active compound that showed a cytotoxicity against cultured human melanoma cells. Betulinic acid was found to be an excellent antitumor compound against human melanoma due to its unique *in vitro* and *in vivo* cytotoxicity profile. Betulinic acid showed a

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strong selective antitumor activity against melanoma by induction of apoptosis.

Betulinic acid also was found to have activity against the other cancer cell lines that were tested. The cytotoxicity of betulinic acid, and its lack of toxicity toward normal cells, afford a favorable therapeutic index. The cell lines evaluated for cytotoxicity were A431 (squamous), BC-1 (breast), ZR-75-1 (hormone-dependent human breast cancers), neuroblastoma, COL-2 (colon), HT-1080 (sarcoma), KB (human oral epidermoid carcinoma), LNCaP (prostate), LU-1 (lung), U373 (glioma), and MEL-1, -2, -3, and -4 (melanoma).

The bark of white birch, *Betula alba*, contains betulin (up to about 25%), lup-20(29)-ene-3 $\beta$ ,28-diol, and betulinic acid (0.025%), but it is difficult to isolate a sufficient quantity of betulinic acid to perform an extensive bioassay. It has been found that a quantity of betulinic acid could be provided from betulin through a simple synthetic approach.

As shown in Table 1, *in vitro* growth of MEL-2 cells was inhibited by betulinic acid, i.e., an ED<sub>50</sub> value of about 2  $\mu$ g/ml. In this particular test, none of the other cancer cell lines tested was affected by betulinic acid (i.e., ED<sub>50</sub> values of greater than 20  $\mu$ g/ml). The cytotoxic response mediated by betulinic acid is not exclusively limited to the MEL-2 melanoma cell line. Dose-response studies performed with additional human melanoma cell lines, designated MEL-1, MEL-3 and MEL-4, demonstrated ED<sub>50</sub> values of 1.1, 3.3 and 4.8  $\mu$ g/ml, respectively.

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As further illustrated in Table 1, other known antitumor agents, such as paclitaxel, camptothecin, ellipticine, homoharringtonine, mithramycin A, podophyllotoxin, vinblastine, and vincristine, demonstrated relatively intense, nonselective cytotoxic activity with no discernible cell-type selectivity.

In the following Table 1, the extracted betulinic acid and the other pure compounds were tested for cytotoxicity against the following cultured human cell lines: A431 (squamous cells), BC-1 (breast), COL-2 (colon), HT-1080 (sarcoma), KB (human oral epidermoid carcinoma), LNCaP (prostate), LU-1 (lung), MEL-2 (melanoma), U373 (glioma) and ZR-75-1 (breast).

TABLE 1

Cytotoxic Activity Profile of the Crude Ethyl Acetate  
Extract Obtained from *Ziziphus mauritiana*, Betulinic acid,  
Other Antineoplastic Agents

Compound	ED <sub>50</sub> (µg/ml)									
	A431	BC-1	COL-2	HT-1080	KB	LNCaP	LU-1	MEL-2	U373	ZR 75-1
<i>Ziziphus mauritiana</i> crude extract	>20	>20	>20	9.5	>20	>20	5.2	3.7	>20	15.8
Betulinic acid	>20	>20	>20	>20	>20	>20	>20	2.0	>20	>20
Taxol	0.00	0.02	0.02	0.00	0.02	0.02	0.00	0.06	0.008	0.02
Camptothecin	0.00	0.07	0.005	0.01	0.00	0.006	0.00	0.02	0.000	0.001
Ellipticine	0.5	0.2	0.3	1.8	0.04	0.8	0.02	0.9	1.6	0.9
Homoharringtonine	0.02	0.03	0.1	0.01	0.00	0.03	0.03	0.04	0.2	0.06
Mithramycin A	0.09	0.3	0.06	1.5	0.09	0.05	0.2	1.2	0.04	0.2
Podophyllotoxin	0.03	0.03	0.005	0.00	0.08	0.04	0.00	0.003	0.004	0.4
Vinblastine	0.05	0.06	0.01	0.02	0.04	0.1	0.02	0.01	1.1	0.3
Vincristine	0.01	0.01	0.02	0.02	0.00	0.1	0.05	0.02	0.06	0.4

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When using the test method used to develop the data in Table 1 (i.e., Method A), the greatest cytotoxic activity in response to betulinic acid was observed against human melanoma cells. Based on the data summarized in Table 1, *in vivo* studies using betulinic acid were performed. As set forth in Table 2, when betulinic acid was tested for cytotoxicity against cancer cell lines using other tests (i.e., Methods B and C), appreciable activity also was observed against other human cancer cell types (e.g., breast, sarcoma, lung, colon, squamous cell, prostate, and glioma). However, the greatest activity was observed against human melanoma cells. Betulinic acid also showed excellent cytotoxic activity against human neuroblastoma cell lines.

As confirmed by the data summarized in Table 1, betulinic acid has been reported as noncytotoxic with respect to cultured KB cells. Cytotoxicity of the crude extracts and purified compounds was determined in a number of cultured human cancer cell lines. Table 1 sets forth the various types of cancer cells evaluated using Method A. The cells were cultured in appropriate media and under standard conditions. To maintain logarithmic growth, the media were changed 24 hours prior to cytotoxic assays. On the day of the assay, the cells were harvested by trypsinization, counted, diluted in media, and added to 96-well plates containing test compounds dissolved in DMSO. The final DMSO concentration was 0.05%.

Table 2 summarizes test data showing the cytotoxicity of betulinic acid using test samples dissolved in tissue culture media (Method B) or 5% aqueous bovine serum albumin (Method C). Methods B and C illustrate the cytotoxicity of betulinic acid

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against cancer cell lines in addition to melanoma, particularly breast cancer, fibrosarcoma, lung cancer, colon cancer, epidermoid carcinoma, hormone-dependent breast cancer, and glioma.

- 5           In each of Methods A-C, the plates were incubated for three days. Following the incubation period, the cells were fixed and stained with sulforhodamine B (SRB) dye. The bound dye was liberated with Tris base, and the OD<sub>515</sub> (optical density
- 10 at 515 nm) was measured on an ELISA reader. The growth of the betulinic acid-treated cells was determined by the OD<sub>515</sub> values, and the growth was compared to the OD<sub>515</sub> values of treated control cells of Methods A-C. Dose response studies were per-
- 15 formed to generate ED<sub>50</sub> values. As used herein, the term OD<sub>515</sub> is defined as optical density at 515 nm, and the term ED<sub>50</sub> is defined as the concentration of a compound required to reduce cell number by 50%.



Table 2

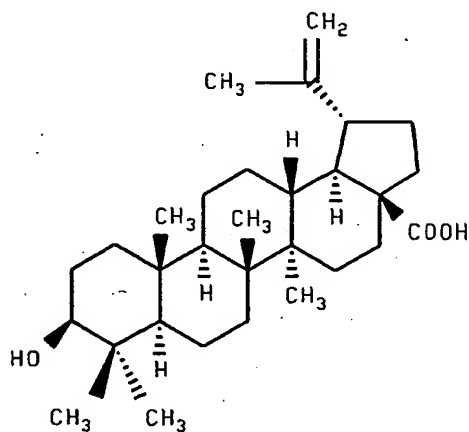
Table 2												
		ED <sub>50</sub> (µg/ml)										
	BCL	HT	Lul	Mel1	Mel2	Mel4	Col2	KB	A431	LNCap	ZA-75-1	U373
Betulinic Acid												
Method A	>20	>20	>20	1.1	1.9	4.8	17.2	19.2	>20	>20	>20	>20
Method B	11.2	11.3	7.7	0.9	0.9	1.6	13.3	>20	12.1	19.3	6.4	12.1
Method C	12.6	10.0	NT	NT	1.6	NT	NT	16.6	>20	16.6	6.9	18.7

Sample dissolved in 10% DMSO (Method A), tissue culture media (Method B), or 5% aqueous bovine serum albumin (Method C); BC1, human breast cancer; HT, human fibrosarcoma; Lu1, human lung cancer; Mell, Mel2, Mel4, human melanoma; Col2, human colon cancer; KB, human oral epidermoid carcinoma; A431, human epidermoid carcinoma; LNCap, human prostate cancer; ZA-75-1, hormone-dependent human breast cancer; U373, human glioma; NT, not tested.

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The present invention, therefore, is directed to a method and composition for preventing or inhibiting tumor growth. The ultimate active compound is betulinic acid or a derivative of betulinic acid, which is generated *in vivo* from a pro-drug of betulinic acid or derivative thereof. Betulinic acid can be isolated by a method comprising the steps of preparing an extract from the stem bark of *Ziziphus mauritiana* and isolating the betulinic acid. Alternatively, betulin can be isolated from the extract and used as a precursor for the synthesis of betulinic acid. The betulinic acid then is optionally converted to a betulinic acid derivative, followed by conversion of betulinic acid or derivative thereof to a prodrug.

Betulinic acid has the structural formula (1):



(1)

Betulinic acid is fairly widespread in the plant kingdom, and some biological activities have been reported.

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Betulinic acid was obtained by extracting a sample of air-dried, milled stem bark (450 g) of *Z. mauritiana* with 80% aqueous methanol. The aqueous methanol extract then was partitioned successively with hexane and ethyl acetate to provide hexane, ethyl acetate, and aqueous extracts. Among these extracts, the ethyl acetate (13.5 g) extract showed cytotoxic activity against a cultured melanoma cell line (MEL-2) with an  $ED_{50}$  of 3.7  $\mu\text{g/ml}$ . The ethyl acetate extract was chromatographed on a silica gel column using hexane-ethyl acetate (4:1 to 1:4) as eluent to give 10 fractions. Fractions 3 and 4 were combined and subjected to further fractionation to afford an active fraction (fraction 16) showing a major single spot by thin-layer chromatography ( $R_f$  0.67:  $\text{CHCl}_3$ :MeOH (chloroform:methanol) (10:1)), which yielded 72 mg of colorless needles after repeated crystallization from methanol (overall yield from dried plant material: 0.016% w/w).

The isolated active compound, betulinic acid ( $ED_{50}$  of 2.0  $\mu\text{g/ml}$  for MEL-2), has a molecular formula of  $\text{C}_{30}\text{H}_{48}\text{O}_3$ , as determined by high-resolution mass spectral analysis, a melting point range of 292-293°C (decomposition). The literature melting point range for betulinic acid is 290-293°C. A mixed melting point range with a known sample of betulinic acid was not depressed. The optical rotation of the compound was measured as +7.3° ( $c=1.2$ ; pyridine) (lit. +7.5°). The identity of the isolated compound as betulinic acid was confirmed by comparing the above physical properties, as well as  $^1\text{H}$ -NMR,  $^{13}\text{C}$ -NMR, and mass spectral data of the isolated compound, with physical data and spectra of a known sample of betulinic acid as reported in the literature.

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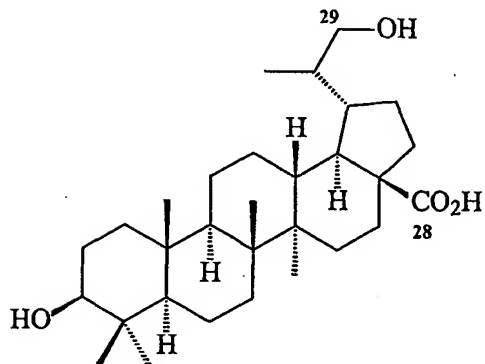
Prodrugs of betulinic acid derivatives also can be used in the composition and method of the present invention. An examination of the structure of betulinic acid reveals that betulinic acid contains several positions, i.e., the C-3, C-20, C-28, C-29, and C-30 positions, where functional groups can be introduced. For example, see Pezzuto et al. U.S. Patent No. 5,962,527, incorporated herein by reference. In addition, the introduced functional groups, if desired, then can be modified. Through a series of reactions at these five positions, a large number of betulinic acid derivatives were prepared and evaluated for bioefficacy against a series of human tumor cell lines, especially against human melanoma cell lines.

It is well established that a prodrug approach, wherein a drug is derivatized into a form suitable for formulation and/or administration, and then is released as the drug *in vivo*, has been successfully employed to transiently (e.g., bioreversibly) alter the physicochemical properties of the drug (see, H. Bundgaard, Ed., *Design of Prodrugs*, Elsevier, Amsterdam, (1985); R.B. Silverman, *The Organic Chemistry of Drug Design and Drug Action*, Academic Press, San Diego, chapter 8, (1992); K.M. Hillgren et al., *Med. Res. Rev.*, 15, 83 (1995)).

Generally, two strategies can be used to increase aqueous solubility of a drug compound: (a) introduction of an ionic or ionizable group and (b) derivatization in such a manner that the prodrug has a decreased melting point (see G.L. Amidon, *Techniques of Solubilization of Drugs*, Yalkowsky et al., Ed., Marcel Dekker, New York, pp. 183-221, (1981)). Both the 3-OH and the carboxylic acid group (C-28)

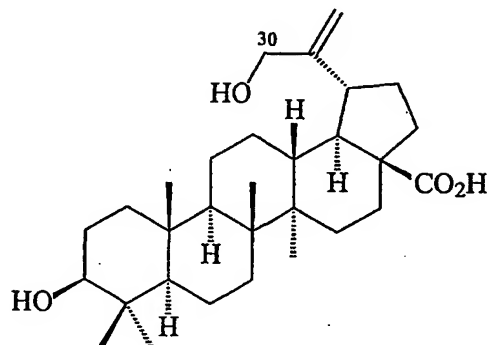
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of betulinic acid are potential positions for such modifications. In addition, introduction of a hydroxy group at either the C-29 (2) or C-30 (3) position allows for similar modifications.



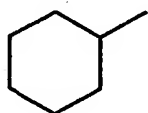
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(2)

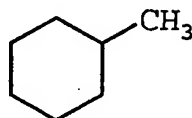


(3)

In the structures herein, for a bond lacking a substituent, the substituent is methyl, for example,



is

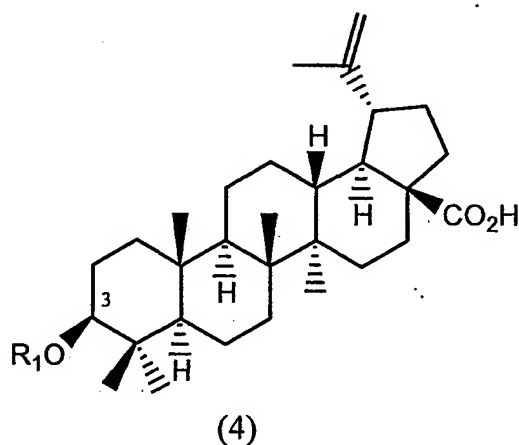


10 When no substituent is indicated as attached to a carbon atom on a ring, it is understood that the carbon atom contains the appropriate number of hydrogen atoms.

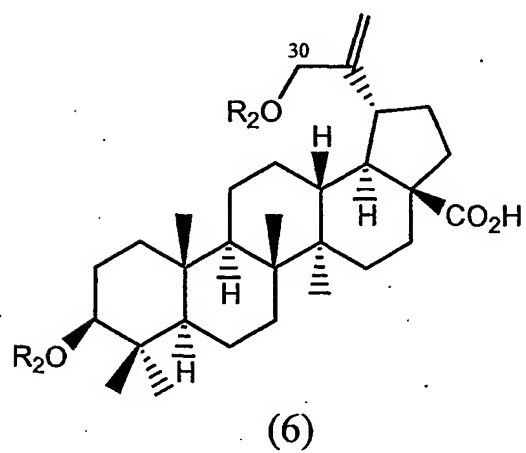
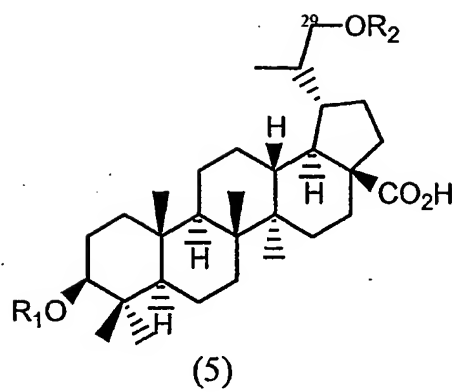
15 Among the ionic or ionizable promoieties, a hemisuccinate, a dialkylaminoacetate, and an amino acid ester are commonly used esters for increasing

- 21 -

aqueous solubility of a hydroxy group-containing drug. Therefore, compounds 4a-4g, 5a-5g, and 6a-6g can be synthesized. Because the susceptibility of esters to undergo chemical and/or enzymatic hydrolysis varies widely, the present compounds, including both anionic and cationic moieties, are expected to be suitable substrates for a variety of endogenous hydrolytic enzymes, such as esterases, and to deliver the parent drug, i.e., betulinic acid or a betulinic acid derivative, bioreversibly.

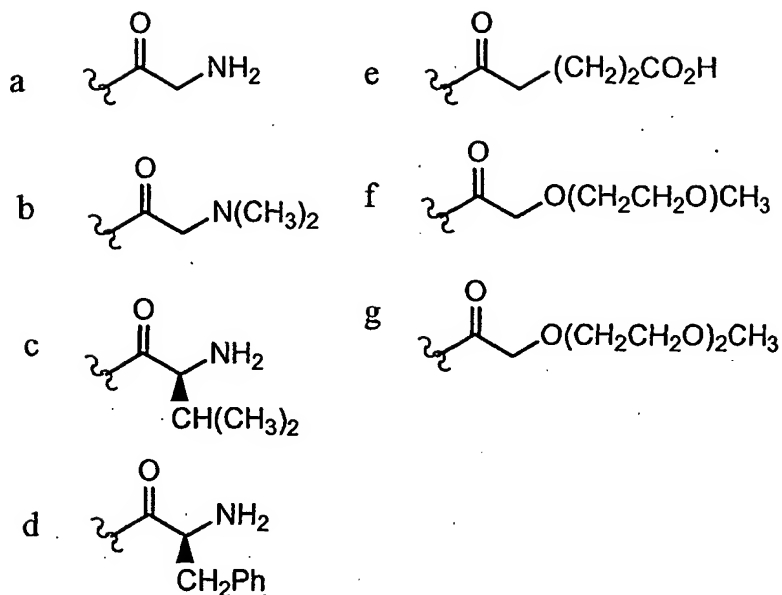


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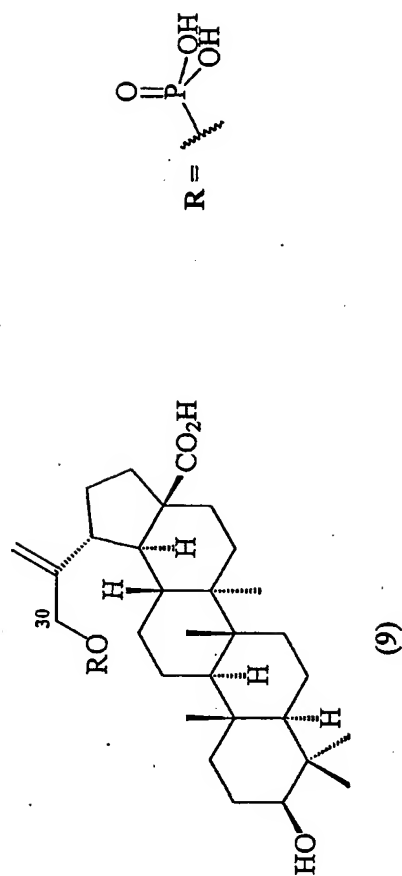
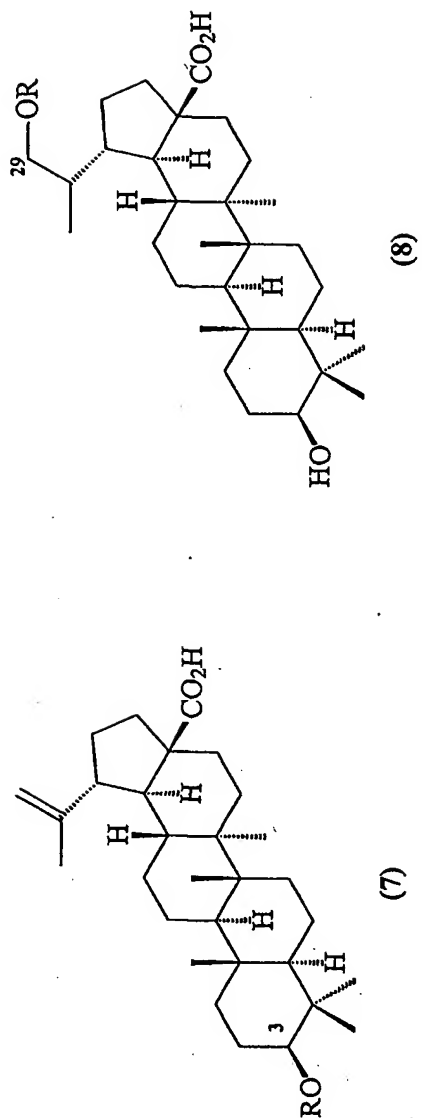
wherein  $\text{R}_1$  and  $\text{R}_2$ , independently, are selected from the group consisting of:

- 23 -



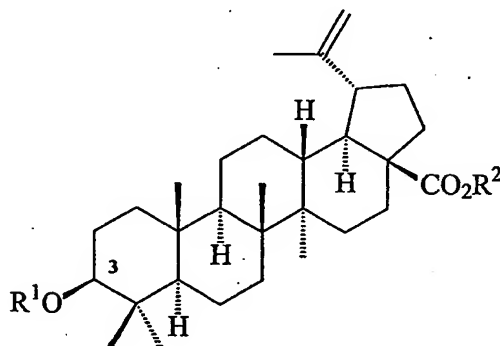
Phosphate prodrugs also can be used to overcome drug delivery problems that can compromise the therapeutic benefits of the parent drug (see, M.G. Nicolaou et al., *J. Org. Chem.*, 61, 8636, (1996), for example). In the presence of alkaline phosphatase, an enzyme widely distributed in a variety of tissues such as the liver, kidney tubules, and intestinal epithelium, for example, phosphomonoesters can undergo hydrolysis to release the parent hydroxy-containing drug and an inorganic phosphate. The phosphate prodrugs 7-9 are designed to be a substrate for the endogenous alkaline phosphatases.





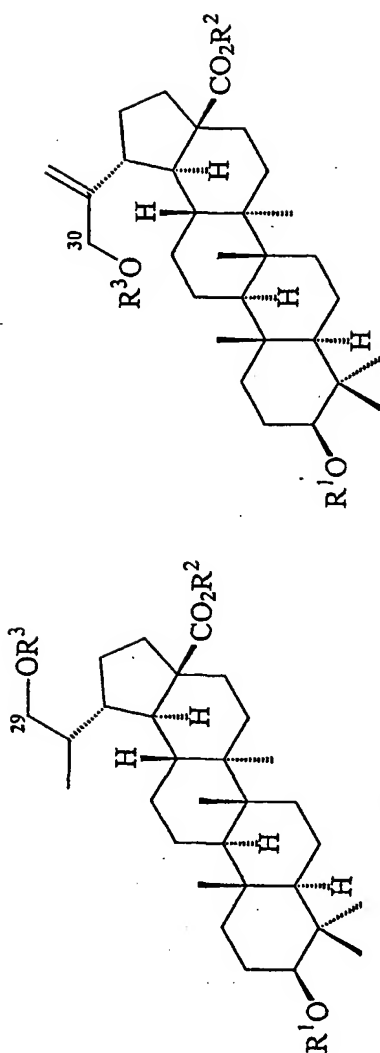
- 25 -

Compounds 10a-10b, 11a-11g, and 12a-12g are pivalyloxymethyl derivatives. This (acyloxy)-alkyl class of prodrugs have proven useful with respect to improving the biological availability of a parent carboxylic acid, as well as alcohols (see, N. Bodor et al., *Int. J. Pharm.*, 7, 63 (1980); and N. Bodor et al., *J. Org. Chem.*, 48, 5280 (1983)). For example, the 3-pivalyloxymethyl ether of 17 $\beta$ -estradiol substantially reduced the melting point of the parent 17 $\beta$ -estradiol. It is theorized, but not relied upon herein, that a pivalyloxymethyl prodrug, after absorption, is hydrolyzed enzymatically by nonspecific esterases to the corresponding hydroxymethyl derivative, which subsequently decomposes spontaneously to betulinic acid (or betulinic acid derivative) and formaldehyde.



(10)

a.  $R^1 = (CH_3)_3CCO_2CH_2$ ,  $R^2 = H$ b.  $R^1 = (CH_3)_3CCO_2CH_2$ ,  $R^2 = (CH_3)_3CCO_2CH_2$



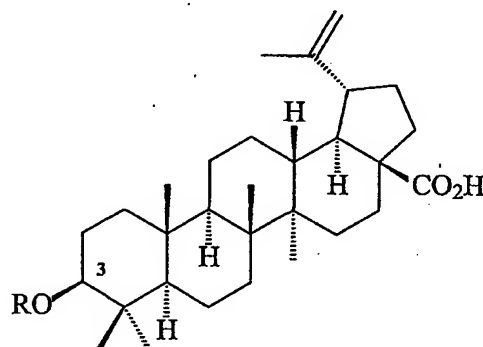
(11)

(12)

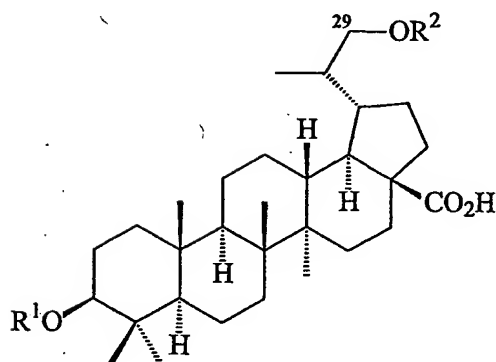
- a.  $\text{R}^1 = (\text{CH}_3)_3\text{CCO}_2\text{CH}_2$ ,  $\text{R}^2 = \text{H}$ ,  $\text{R}^3 = \text{H}$
- b.  $\text{R}^1 = \text{H}$ ,  $\text{R}^2 = (\text{CH}_3)_3\text{CCO}_2\text{CH}_2$ ,  $\text{R}^3 = \text{H}$
- c.  $\text{R}^1 = \text{H}$ ,  $\text{R}^2 = \text{H}$ ,  $\text{R}^3 = (\text{CH}_3)_3\text{CCO}_2\text{CH}_2$
- d.  $\text{R}^1 = (\text{CH}_3)_3\text{CCO}_2\text{CH}_2$ ,  $\text{R}^2 = (\text{CH}_3)_3\text{CCO}_2\text{CH}_2$ ,  $\text{R}^3 = \text{H}$
- e.  $\text{R}^1 = (\text{CH}_3)_3\text{CCO}_2\text{CH}_2$ ,  $\text{R}^2 = \text{H}$ ,  $\text{R}^3 = (\text{CH}_3)_3\text{CCO}_2\text{CH}_2$
- f.  $\text{R}^1 = \text{H}$ ,  $\text{R}^2 = (\text{CH}_3)_3\text{CCO}_2\text{CH}_2$ ,  $\text{R}^3 = (\text{CH}_3)_3\text{CCO}_2\text{CH}_2$
- g.  $\text{R}^1 = (\text{CH}_3)_3\text{CCO}_2\text{CH}_2$ ,  $\text{R}^2 = (\text{CH}_3)_3\text{CCO}_2\text{CH}_2$ ,  $\text{R}^3 = (\text{CH}_3)_3\text{CCO}_2\text{CH}_2$

- 27 -

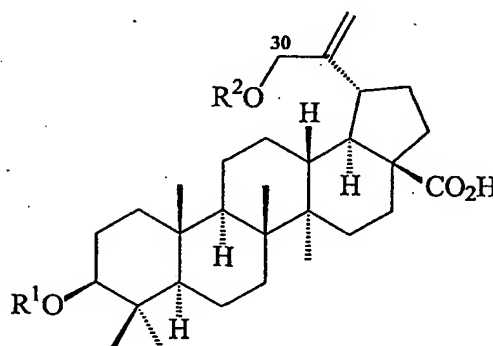
- Another class of prodrugs of the present invention is sulfates of betulinic acid (13) and betulinic acid derivatives (14a-c, 15a-c). The sulfate moiety increases the hydrophilicity of the compounds, while endogenous nonspecific sulfatases release betulinic acid or a betulinic acid derivative and an inorganic sulfate *in vivo*.



(13)

R = SO<sub>3</sub>H

(14)



(15)

- a. R<sup>1</sup> = SO<sub>3</sub>H, R<sup>2</sup> = H  
 b. R<sup>1</sup> = H, R<sup>2</sup> = SO<sub>3</sub>H  
 c. R<sup>1</sup> = SO<sub>3</sub>H, R<sup>2</sup> = SO<sub>3</sub>H

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Synthesis of Betulinic Acid

Even though betulinic acid has been isolated from several genus of higher plants, the abundance of betulinic acid is sufficiently low such that it is tedious to isolate in a useable quantity (see, T. Fujioka et al., *J. Nat. Prod.*, 57, 243-7 (1994); S. Siddiqui et al., *J. Nat. Prod.*, 57, 243-7 (1994); and F. Robinson et al., *Phytochemistry*, 9, 907-9 (1970)). However, as noted previously, betulin 16, is readily available from the bark of white birch, in concentrations up to about 25% by weight, and hundreds of tons of the bark are discarded each year in processes such as furniture manufacture.

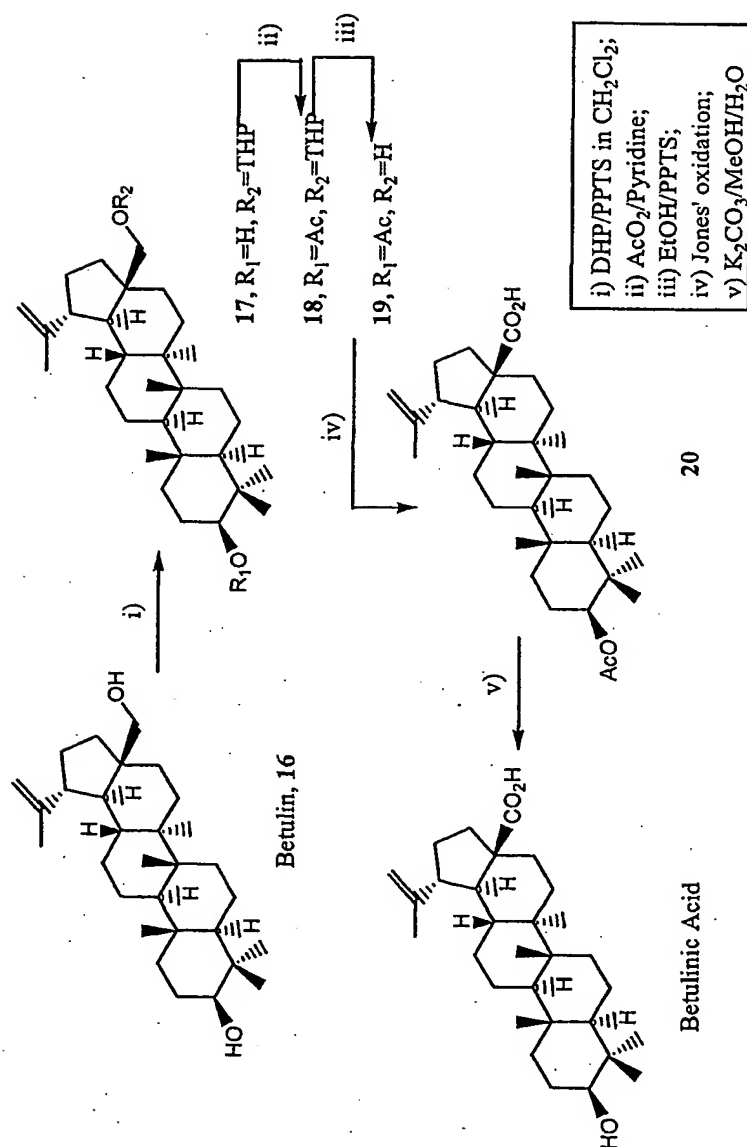
Two synthetic methods for the production of betulinic acid from betulin have been reported. One method involves three steps with an overall 71% yield, and the second method is a five-step process affording an overall 55% yield (see D.S.H.L. Kim et al., *Synth. Commun.*, 27, 1607-1612 (1997)). The first method produced a higher overall yield in the laboratory, but the product is difficult to purify during scale up because the undesirable 3 $\alpha$ -isomer was formed as a by-product. The second method, although providing a lower yield, does not produce the undesired 3 $\alpha$ -isomer, and involves predominantly protecting and deprotecting procedures, and thus is preferred for the synthesis of betulinic acid.

Therefore, betulin 16 can be isolated from the bark of *Betula alba*, following a literature method, and converted to betulinic acid as shown in following Scheme 1. Briefly, the primary hydroxy group of betulin is selectively protected as a tetrahydropyran (THP) ether 17 in step i), then the secondary hydroxy group is acetylated to provide

- 29 -

compound 18 in step ii). After the THP group in compound 18 is selectively removed in step iii), the resulting primary alcohol 19 is oxidized under Jones oxidation conditions ( $\text{CrO}_3/\text{H}_2\text{SO}_4/\text{acetone}/0^\circ\text{C}$ ) in step  
5 iv) to provide carboxylic acid 20. The acetyl group finally is removed under mild basic conditions in step v) to yield betulinic acid.

Scheme 1

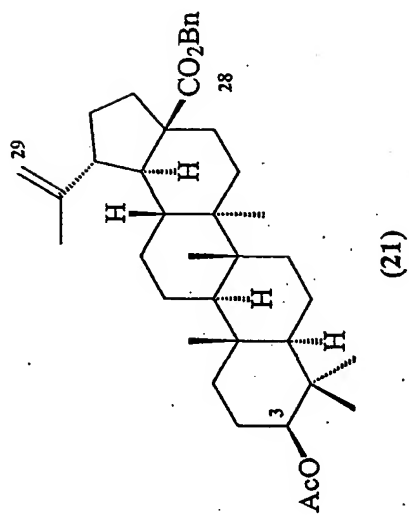
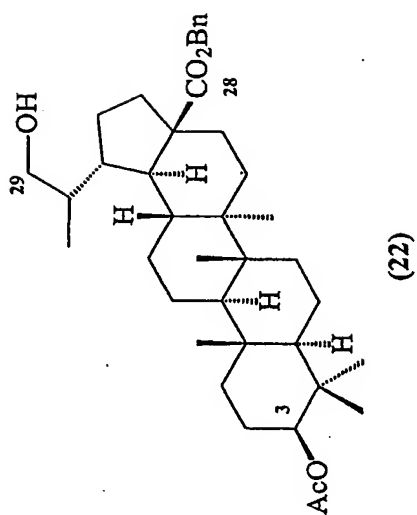


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Synthesis of C-29 and C-30 Betulinic Acid Alcohols

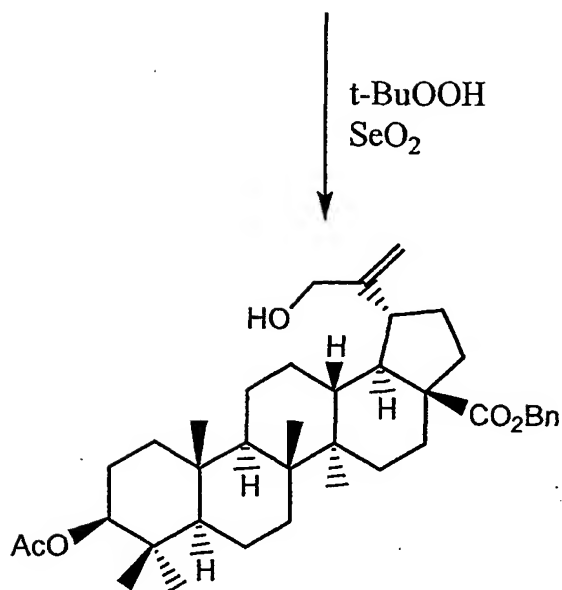
The synthesis of betulinic acid derivatives 2 and 3 is accomplished by hydroboration and allylic oxidation, respectively. The C-29 alcohol is synthesized from the common intermediate 20, with additional protection of the carboxylic acid as the benzyl ester (21). Hydroboration of (21) with  $\text{BH}_3$ -dimethyl sulfide, followed by oxidation under basic conditions yields the C-29 alcohol (22), which then is selectively functionalized without interference of the C-3 alcohol or C-28 carboxylic acid. Hydrolysis of the C-3 acetate followed by catalytic hydrogenolysis of the C-28 benzyl ester yields the unprotected C-29 alcohol.





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(21)



Synthesis of the C-30 allylic alcohol (3) is achieved by reaction of the protected betulinic acid (21), followed by oxidation with catalytic  
5 selenium dioxide and t-butyl hydroperoxide. The protected C-30 allylic alcohol (23) can be further manipulated as desired, and the C-3 acetate and C-28 benzyl ester can be removed by hydrolysis with potassium carbonate.

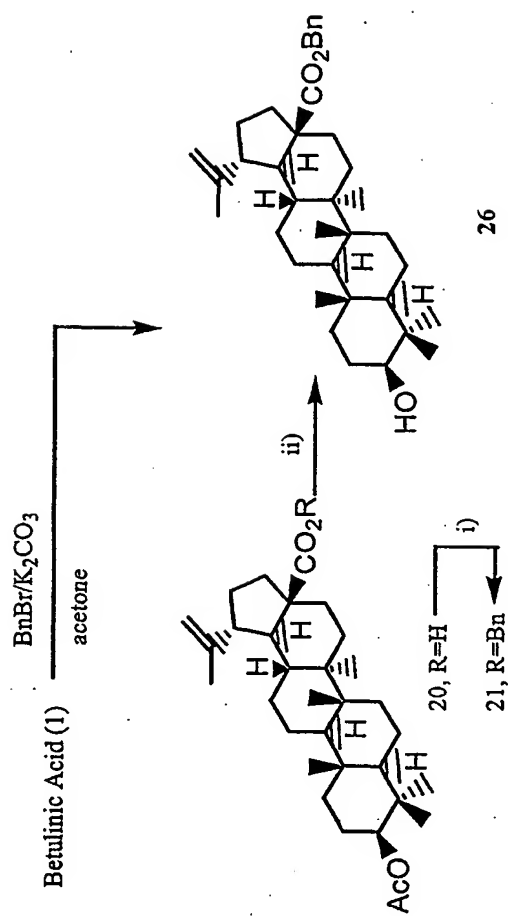
10 To synthesize prodrugs 2a-g, the carboxylic acid group in betulinic acid is protected. A variety of protecting groups can be employed. Because the ester group in the present prodrugs may be labile, a protecting group capable of being removed  
15 under very mild conditions is used. One preferred protecting agent is a benzyl group because a benzyl

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group is readily cleaved by hydrogenolysis under neutral conditions. The 20(29)-ene double bond in betulinic acid can be saturated during hydrogenolysis. However, the benzyl group can be cleaved in the presence of a double bond by using  $t\text{-BuMe}_2\text{SiH}$  and  $\text{Pd}(\text{OAc})_2$  (see, M. Sakaitgani et al., *Tetrahedron Lett.*, 27, 3753 (1986)). Therefore, the benzyl ester of betulinic acid 26 is prepared from the acetate 20. Reaction of acetate 20 with benzyl bromide in the presence of potassium carbonate ( $\text{K}_2\text{CO}_3$ ) in acetone yields the double ester 21, which is deacetylated using  $\text{K}_2\text{CO}_3$  in methanol (MeOH) as described above, providing precursor 26 (Scheme 2).

Alternatively, a direct, one-step benzylation of betulinic acid using benzyl bromide in the presence of  $\text{K}_2\text{CO}_3$  in acetone yielded precursor 26 in an acceptable yield (Scheme 2).

Scheme 2



i)  $\text{BnBr}/\text{K}_2\text{CO}_3/\text{acetone}$ ; 2)  $\text{K}_2\text{CO}_3/\text{MeOH}/\text{H}_2\text{O}$

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Synthesis of Betulinic Acid Ester Prodrugs

Compounds (4), (5), and (6a-d) are amino acid esters synthesized according to the conventional methods. Thus, when activated by carbonyldiimidazole (CDI) or dicyclohexylcarbodiimide (DCC) in refluxing tetrahydrofuran (THF), benzyl ester 26 reacts with N,N-dimethylglycine, or Cbz-protected amino acids, such as glycine, L-leucine and L-phenylalanine, respectively (Scheme 3). Cbz is an abbreviation for carboxybenzoyl. The formed esters 27a-d are treated with t-butyldimethylsilane (t-BuMe<sub>2</sub>SiH) in the presence of Pd(OAc)<sub>2</sub> to cleave the benzyl as well as Cbz-protecting groups, resulting in the formation of compounds (4a-d). The same scheme can be used to synthesize compounds (4e), (4f), (5a-f), and (6a-f) via (27a-f), respectively. All the carboxylic acids employed are commercially available.

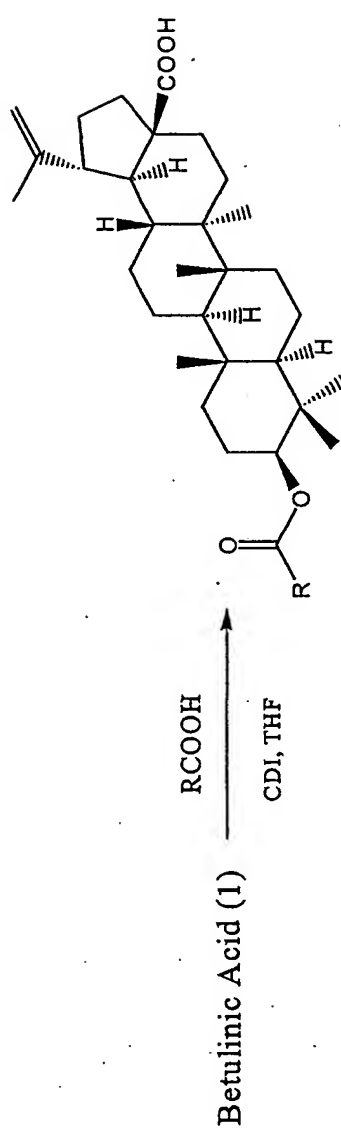
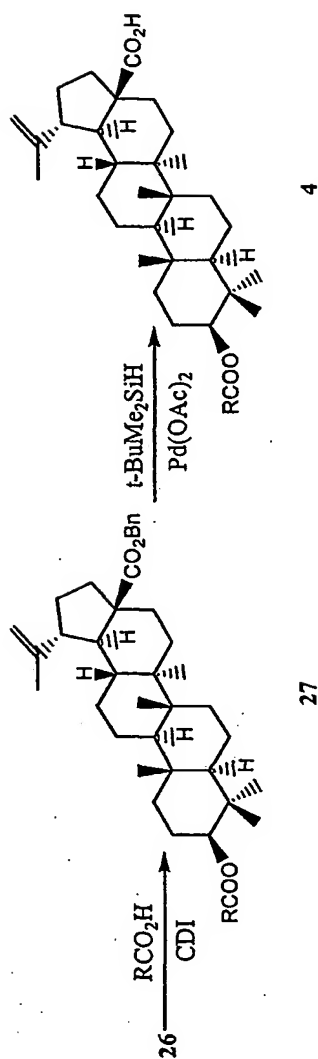
Alternatively, free acid analogues (4) can be synthesized directly from betulinic acid (Scheme 3). Boc-Gly-OH was activated with CDI at room temperature for 2 hours, then refluxed with betulinic acid overnight to provide (4i) in 97% yield. Boc is an abbreviation for t-butylcarboxy. Deprotection of the Boc group with trifluoroacetic acid/dichloromethane (TFA/DCM) resulted in rearrangement of the betulinic acid ring system. After trying several methods, it was found that 4M HCl in dioxane cleaves the Boc group without attacking the betulinic acid ring system.

By a similar method, products (4f) and (4j) were synthesized according to synthetic scheme 3.

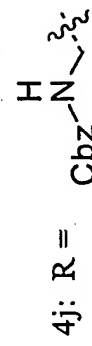
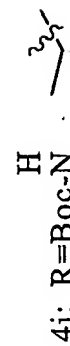
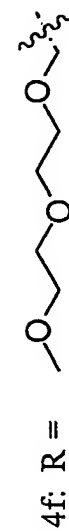
- 37 -

The hemisuccinate derivative (4g) was prepared according to a literature method (L. Colla et al., *J. Med. Chem.*, 26, 602 (1983)) by reacting benzyl ester 26 with succinic anhydride in the presence of triethylamine in DMF, followed by de-benzylation (Scheme 4). This scheme also is used to prepare derivatives (5g) and (6g).

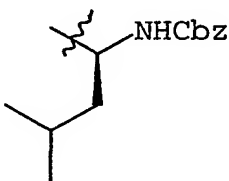
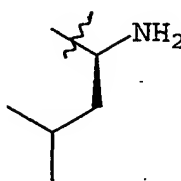
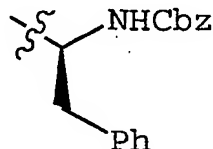
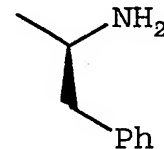
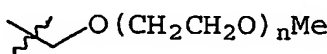
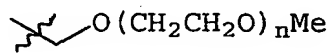
### Scheme 3



(4f, 4i, 4j)



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R in 27	R in 4
CH <sub>2</sub> NHCbz (27a)	CH <sub>2</sub> NH <sub>2</sub> (4a)
CH <sub>2</sub> NMe <sub>2</sub> (27b)	CH <sub>2</sub> NMe <sub>2</sub> (4b)
 (27c)	 (4c)
 (27d)	 (4d)
 (n=1,2) (27 e, f)	 (n=1,2) (4 e, f)

Cbz = carboxybenzoyl

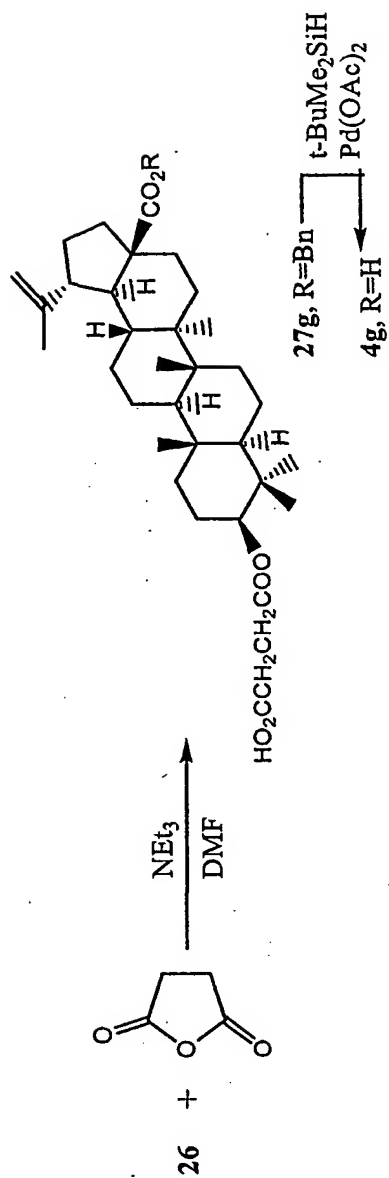
Me = methyl

Ph = phenyl



- 40 -

Scheme 4

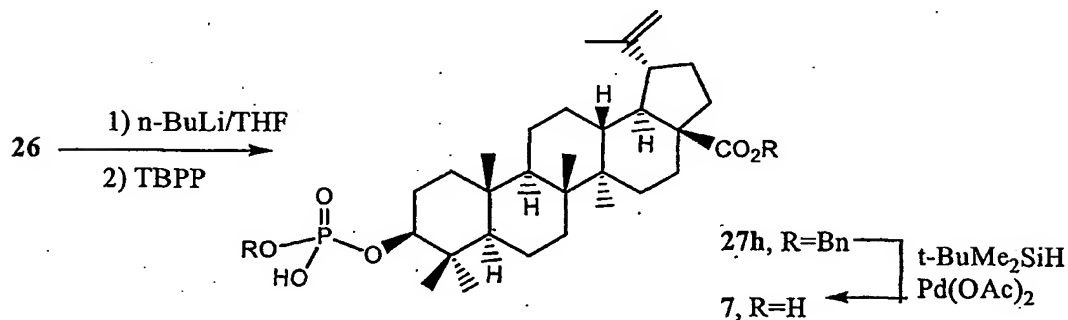


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Synthesis of Phosphate Prodrugs (7-9)

The commercially available tetrabenzylpyrophosphate (TBPP) is a suitable phosphorylating agent. Thus, treatment of benzyl ester 26 with butyllithium in THF, followed by TBPP, can afford phosphate diester (7) after aqueous workup (Scheme 5). Debenzylation is achieved by treatment with *t*-BuMe<sub>2</sub>SiH in the presence of Pd(OAc)<sub>2</sub>, providing phosphoric carboxylic acid (7). This route is also used for derivatives (8) and (9).

Scheme 5

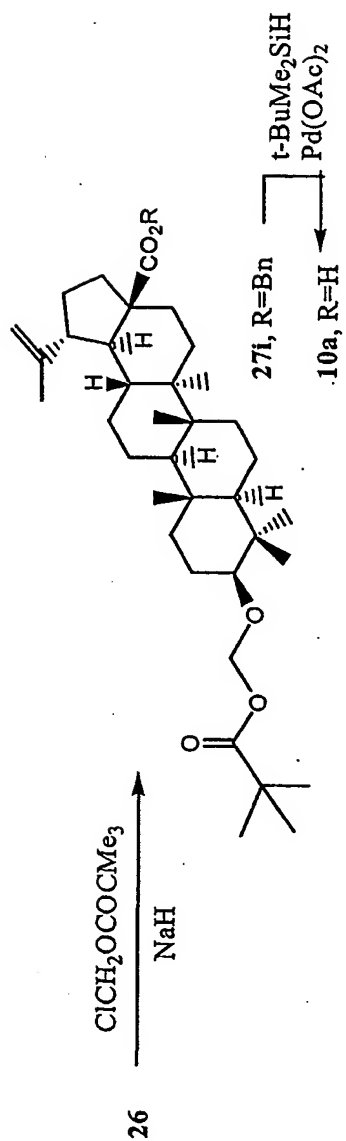
Synthesis of Acyloxyalkyl Derivatized Prodrugs (10-12)

Chloromethyl pivalate is commercially available, and other chloromethyl esters can be prepared by published literature methods (see, Eurato et al., *Acta Chem. Scand.*, 20, 1276 (1966)). Reaction of the sodium or lithium salt of benzyl ester 26 with chloromethyl pivalate provides pivaloyloxy-methyl derivative 27i (Scheme 6). Debenzylation of 27i with *t*-BuMe<sub>2</sub>SiH and Pd(OAc)<sub>2</sub> produces the corre-

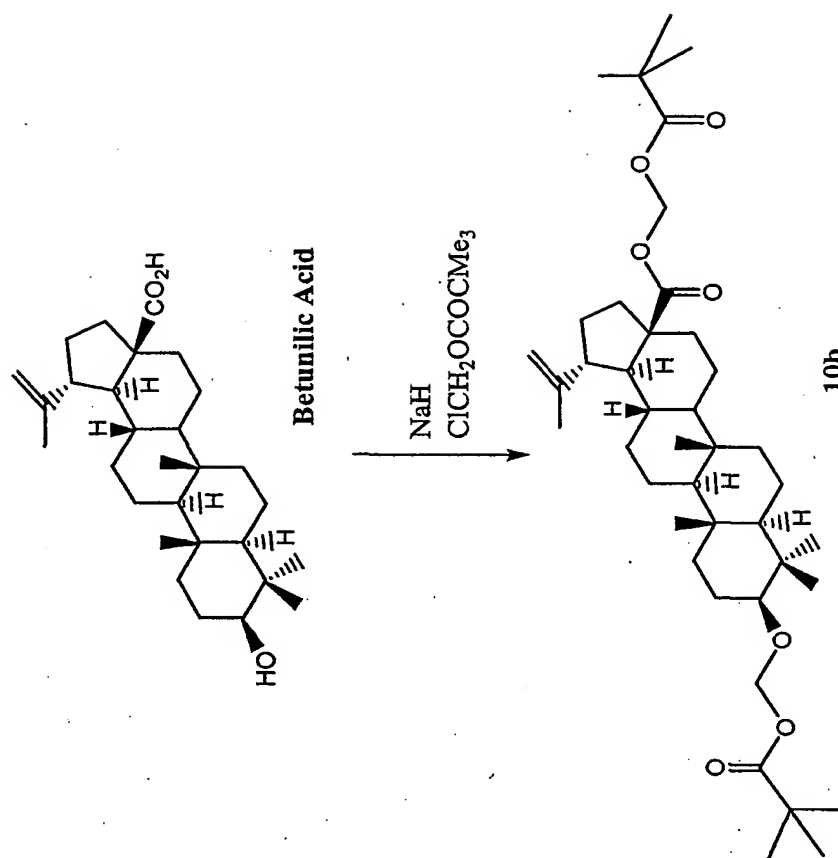
- 42 -

sponding monopivaloyloxymethyl compound 10a. Treatment of the disodium salt of betulinic acid with chloromethyl pivalate furnishes the bis-pivaloyloxymethyl compound 10b (Scheme 6). The literature reports that chloromethyl esters may not be sufficiently reactive, and, accordingly, can be converted to the corresponding iodo analogues by treatment with NaI.

Scheme 6



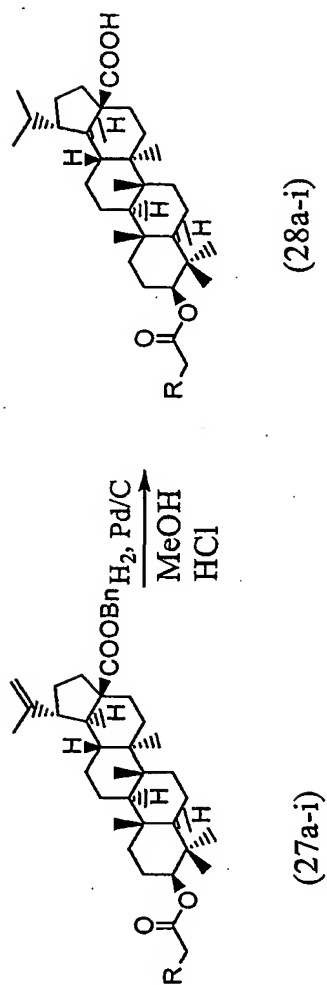
- 44 -



- 45 -

Hydrogenolysis of benzylesters (27a)-(27i) yielded the corresponding 20(29)-saturated betulinic acid (28a)-(27i) (Scheme 7).

Scheme 7



- 47 -

The following abbreviations are used in the synthetic procedures:  $K_2CO_3$  (potassium carbonate),  $MgSO_4$  (magnesium sulfate), EtOAc (ethyl acetate), THF (tetrahydrofuran), NaH (sodium hydride),  
5 HCl (hydrochloric acid), and  $Et_2O$  (diethyl ether).

#### Synthesis of benzyl-protected betulinic acid (26)

Betulinic acid (9.76 g, 21.41 mmole) and  $K_2CO_3$  (4.37 g, 31.62 mmole) in acetone (500 ml) was stirred for 20 minutes. Then, benzyl bromide (6.4  
10 ml, 53.81 mmole) was added, and the resulting mixture was stirred at room temperature overnight. Additional benzyl bromide (4.0 ml, 33.63 mmole) was added, and the mixture was stirred for an additional 24 hours at room temperature. The solvent was evaporated, water was added to the residue, then extracted with ethyl acetate. The EtOAc extracts were  
15 washed with brine, and dried over anhydrous  $MgSO_4$ . After removal of solvent, the crude product was purified by silica column using 10% EtOAc/hexane as an eluent to give 7.55 g (64% yield) of product as  
20 white solid.  $^1H$  NMR ( $CDCl_3$ )  $\delta$ : 0.6-1.7 (m, 38H), 1.8-1.9 (m, 2H), 2.1-2.35 (m, 2H), 2.95-3.05 (m, 1H), 3.1-3.2 (m, 1H), 4.59 (d,  $J=2$  Hz, 1H), 4.72 (d,  $J=2$  Hz, 1H), 5.12 (m, 2H), 7.3-7.4 (m, 5H). MS  
25 (APCI $^+$ ): 547.2 (M+1), 529.2 (M- $H_2O$ ). FT-IR ( $cm^{-1}$ ): 3553, 2940, 2866, 1692, 1451.

#### Synthesis of Cbz-Gly ester derivative (27a)

To a solution of Cbz-Gly-OH (209 mg, 1 mmol) in THF (10 ml) was added CDI (162 mg, 1 mmol).  
30 The mixture was stirred at room temperature for 30 minutes, then betulinic acid benzyl ester (26) (238



- 48 -

mg, 0.44 mmol) was added. The resulting mixture was refluxed for one day. The reaction mixture then was diluted with water and extracted three times with EtOAc. The organic layer was washed with brine, and  
5 dried over  $\text{MgSO}_4$ . After removal of solvent, the residue was purified by a column chromatography on silica gel with EtOAc/hexane as an eluent to give 130 mg of Cbz-Gly- betulinic acid ester derivative (27a). Yield:41%. m.p.:55-57°C.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ :  
10 0.6-1.7 (m, 38H), 1.8-2.0 (m, 2H), 2.1-2.35 (m, 2H), 2.95-3.1 (m, 1H), 3.95 (d,  $J=5.4$  Hz, 2H), 4.55 (m, 1H), 4.59 (s, 1H), 4.72 (s, 1H), 5.12 (m, 4H), 5.3 (br, 1H), 7.3-7.4 (m, 10H). MS (ESI $^+$ ): 755.6 ( $\text{M}+\text{NH}_4$ ). FT-IR ( $\text{cm}^{-1}$ ): 3425, 2943, 2869, 1722,  
15 1453. Anal. Calcd for  $\text{C}_{47}\text{H}_{63}\text{N}_1\text{O}_6$ : C, 76.49; H, 8.60; N, 1.90; Found: C, 75.38; H, 8.76; N, 1.83.

Synthesis of Cbz-Phe ester derivative (27d)

Compound (27d) was synthesized by reacting Cbz-Phe-OH with the betulinic acid benzyl ester (26)  
20 by a similar method as described to prepare compound (27a). Yield:15%. m.p. 67-71°C.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 0.70-1.71 (m, 38 H), 1.80-1.95 (m, 2H), 2.1-2.3 (m, 2H), 2.95-3.15 (m, 3H), 4.4-4.5 (m, 1H), 4.6-4.65 (m, 1H), 4.59 (d,  $J=2$  Hz, 1H), 4.72 (d,  $J=2$  Hz, 1H),  
25 5.12 (m, 4H), 5.22 (d,  $J=8$  Hz, 1H), 7.1-7.4 (m, 15H). MS (ESI $^+$ ): 845.6 ( $\text{M}+\text{NH}_4$ ), 828.5 ( $\text{M}+1$ ). FT-IR ( $\text{cm}^{-1}$ ): 3431, 2944, 2868, 1722, 1497. Anal. Calcd for  $\text{C}_{54}\text{H}_{69}\text{N}_1\text{O}_6$ : C, 78.32; H, 8.40; N, 1.69; Found: C, 78.01; H, 8.52; N, 1.61.

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Synthesis of Cbz-Leu ester derivative (27c)

Compound 27c was synthesized by reacting Cbz-Leu-OH with the betulinic acid benzyl ester (26) by a similar method as described to prepare compound (27a). Yield: 6%. m.p. 64-69°C.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 0.7-1.8 (m, 47H), 1.8-2.0 (m, 2H), 2.1-2.3 (m, 2H), 2.95-3.1 (m, 1H), 4.3-4.4 (m, 1H), 4.45-4.55 (m, 1H), 4.59 (d,  $J=2$  Hz, 1H), 4.72 (d,  $J=2$  Hz, 1H), 5.08-5.20 (m, 4H), 7.3-7.4 (m, 10H). MS (APCI $^+$ ): 812.4 ( $\text{M}+\text{NH}_4$ ), 794.7 ( $\text{M}+1$ ). FT-IR ( $\text{cm}^{-1}$ ): 3355, 2946, 2868, 1722, 1453. Anal. Calcd for  $\text{C}_{51}\text{H}_{71}\text{N}_1\text{O}_6$ : C, 77.14; H, 9.01; N, 1.76; Found: C, 77.36; H, 9.11; N, 1.69.

Synthesis of 2-(2-Methoxyethoxy)acetic acid ester derivative (27e)

Compound (27e) was synthesized by reacting 2-(2-methoxyethoxy)acetic acid with the betulinic acid benzyl ester (26) by a similar method as described to prepare compound (27a). Yield: 38%.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 0.75-1.7 (m, 38H), 1.85-1.95 (m, 2H), 2.1-2.3 (m, 2H), 2.95-3.10 (m, 1H), 3.38 (s, 3H), 3.58 (m, 2H), 3.72 (m, 2H), 4.13 (s, 2H), 4.5-4.6 (m, 1H), 4.59 (s, 1H), 4.72 (s, 1H), 5.12 (m, 2H), 7.3-7.4 (m, 5H). MS (ESI $^+$ ): 680.6 ( $\text{M}+\text{NH}_4$ ). FT-IR ( $\text{cm}^{-1}$ ): 2945, 2867, 1754, 1715, 1453. Anal. Calcd for  $\text{C}_{42}\text{H}_{62}\text{O}_6$ : C, 76.09; H, 9.43; Found: C, 76.28; H, 9.47.

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Synthesis of 2-[2-(2-Methoxyethoxy)ethoxy]acetic acid ester derivative (27f)

Compound (27f) was synthesized by reacting 2-[2-(2-methoxyethoxy)ethoxy]acetic acid with the  
5 betulinic acid benzyl ester (26) by a similar method as described to prepare compound (27a). Yield: 52%.  
 $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 0.75-1.7 (m, 38H), 1.85-1.95 (m, 2H),  
2.05-2.3 (m, 2H), 2.95-3.10 (m, 1H), 3.38 (s, 3H),  
3.57 (m, 2H), 3.6-3.75 (m, 6H), 4.13 (s, 2H), 4.5-  
10 4.6 (m, 1H), 4.59 (d,  $J=2$  Hz, 1H), 4.72 (d,  $J=2$  Hz, 1H), 5.12 (m, 2H), 7.3-7.4 (m, 5H). MS (APCI $^+$ ):  
724.3 ( $\text{M}+\text{NH}_4$ ). FT-IR ( $\text{cm}^{-1}$ ): 2942, 2869, 1724, 1454. Anal. Calcd for  $\text{C}_{44}\text{H}_{66}\text{O}_6$ : C, 74.75; H, 9.41; Found: C, 73.72; H, 9.34.

15 Synthesis of pivaloyoxymethyl ether derivative (27i)

To a solution of betulinic acid benzyl ester (26) (526 mg, 0.96 mmol) in THF (10 ml) was added NaH (60%, 66 mg, 1.6 mmol). The mixture was  
20 stirred at room temperature for 1 hour, then chloromethyl pivalate (635 mg, 0.96 mmol) was added. The resulting mixture was stirred at room temperature overnight. The reaction mixture then was diluted with water, and extracted three times with ethyl  
25 acetate. The organic layer was washed with brine, and dried over  $\text{MgSO}_4$ . After removal of solvent, the residue was purified by column chromatography on silica gel with EtOAc/hexane as an eluent to give  
102 mg of the desired product (27i). Yield: 16%.  
30 m.p.: 122-128°C.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 0.7-1.7 (m, 47H), 1.85-1.95 (m, 2H), 2.1-2.3 (m, 2H), 2.95-3.15 (m, 2H), 4.59 (d,  $J=2$  Hz, 1H), 4.72 (d,  $J=2$  Hz, 1H), 5.12 (m, 2H), 5.27 (d,  $J=6$  Hz, 1H), 5.35 (d,  $J=6$  Hz,

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1H), 7.3-7.4 (m, 5H). MS (ESI<sup>+</sup>): 678.7 (M+NH<sub>4</sub>).  
FT-IR (cm<sup>-1</sup>): 2947, 2866, 1746, 1714, 1480. Anal.  
Calcd for C<sub>43</sub>H<sub>64</sub>O<sub>5</sub>: C, 78.14; H, 9.76; Found: C,  
78.46; H, 10.49.

5 Synthesis of Boc-Gly ester derivative (4i)

To a solution of Boc-Gly-OH (525 mg, 3 mmol) in THF (5 ml) was added CDI (486 mg, 3 mmol). The mixture was stirred at room temperature for 2 hours, then betulinic acid (684 mg, 1.5 mmol) was  
10 added. The resulting mixture was refluxed overnight. After removal of solvent, the residue was dissolved in dichloromethane, washed with water, followed by brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. After  
15 removal of solvent, the residue was purified by a column chromatography on silica gel with EtOAc/-hexane as an eluent to give 900 mg of Boc-Gly- betulinic acid derivative (4i). Yield:97%. m.p.: 135-136°C. <sup>1</sup>H NMR (DMSO-d<sub>6</sub>) δ: 0.75-1.7 (m, 47H), 1.75-1.9 (m, 2H), 2.05-2.3 (m, 2H), 2.9-3.0 (m, 1H), 3.63  
20 (d, J=6 Hz, 2H), 4.4 (m, 1H), 4.56 (d, J=2 Hz, 1H), 4.69 (d, J=2 Hz, 1H), 7.18 (t, J=6 Hz, 1H), 12.05 (s, 1H). MS (ESI<sup>-</sup>): 612.4 (M-1). FT-IR (cm<sup>-1</sup>): 2943, 2869, 1702. Anal. Calcd for C<sub>37</sub>H<sub>59</sub>N<sub>1</sub>O<sub>6</sub>: C, 72.39; H, 9.69; N, 2.28; Found: C, 72.13; H, 10.07;  
25 N, 2.20.

Synthesis of Cbz-Gly ester derivative (4j)

Compound (4j) was synthesized by reacting Cbz-Gly-OH with the betulinic acid by a similar method as described to prepare compound (IIIb).  
30 Yield:7%. m.p.:82-88°C. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ: 0.75-1.7 (m, 38H), 1.95-2.05 (m, 2H), 2.1-2.3 (m, 2H), 2.95-

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3.05 (m, 1H), 3.97 (d, J=6 Hz, 2H), 4.5-4.6 (m, 1H),  
4.60 (s, 1H), 4.75 (s, 1H), 5.12 (s, 2H), 5.25 (m,  
1H), 7.3-7.4 (m, 5H). MS (APCI<sup>-</sup>): 646.9 (M+NH<sub>4</sub>).  
FT-IR (cm<sup>-1</sup>): 2943, 2869, 1708. Anal. Calcd for  
5 C<sub>40</sub>H<sub>57</sub>N<sub>1</sub>O<sub>6</sub>: C, 74.15; H, 8.87; N, 2.16; Found: C,  
71.57; H, 8.67; N, 2.22.

**Synthesis of 2-[2-(2-Methoxyethoxy)ethoxy]acetic  
acid ester derivative (4f)**

Compound (4f) was synthesized by reacting  
10 2-[2-(2-methoxyethoxy)ethoxy] acetic acid with the  
betulinic acid by a similar method as described to  
prepare compound (IIIb). Yield: 52%. <sup>1</sup>H NMR (CDCl<sub>3</sub>)  
δ: 0.8-1.7 (m, 38H), 1.9-2.0 (m 2H), 2.1-2.3 (m,  
2H), 2.95-3.05 (m, 1H), 3.38 (s, 3H), 3.57 (m, 2H),  
15 3.6-3.75 (m, 6H), 4.13 (s, 2H), 4.5-4.6 (m, 1H),  
4.61 (d, J=2 Hz, 1H), 4.74 (d, J=2 Hz, 1H). MS  
(APCI<sup>-</sup>): 615.8 (M-1). FT-IR (cm<sup>-1</sup>): 2937, 2869,  
1729, 1694. Anal. Calcd for C<sub>37</sub>H<sub>60</sub>O<sub>6</sub>: C, 72.04; H,  
9.80; Found: C, 71.98; H, 9.90.

20 **Synthesis of Gly ester derivative (4a)**

Compound (4a) (61 mg, 0.1 mmol) was dis-  
solved in 4M HCl dioxane solution and stirred at  
room temperature for 10 min. The reaction mixture  
was diluted with toluene (3 ml). After removal of  
25 solvent under vacuum, the residue was washed with  
Et<sub>2</sub>O to afford 25 mg of the desired product as a  
white solid. Yield: 45%. m.p.: 275°C (dec). <sup>1</sup>H NMR  
(DMSO-d<sub>6</sub>) δ: 0.8-1.7 (m, 38H), 1.75-1.9 (m 2H),  
2.1-2.3 (m, 2H), 2.9-3.0 (m, 1H), 3.8-3.9 (m, 2H),  
30 4.5-4.6 (m, 1H), 4.56 (s, 1H), 4.69 (s, 1H), 8.30  
(br, 3H). MS (APCI<sup>-</sup>): 514.6 (M+1). FT-IR (cm<sup>-1</sup>):  
2941, 2869, 1740. Anal. Calcd for C<sub>32</sub>H<sub>52</sub>ClNO<sub>4</sub>: C,

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69.85; H, 9.53; N, 2.55; Found: C, 66.31; H, 9.38; N, 2.68.

Synthesis of Gly ester saturated derivative (28a)

Compound (27a) (180 mg, 0.28 mmol) was  
5 dissolved in EtOAc (5 ml), diluted with methanol (10 ml), and one drop of HCl solution (6N) was added. The reaction mixture was hydrogenated with 50 mg of 10% palladium on activated carbon as a catalyst at 50 psi hydrogen pressure at room temperature for 4  
10 hrs. The catalyst was removed by filtration. After removal of solvent, the residue was washed with Et<sub>2</sub>O to afford 110 mg of the desired product as a white solid. Yield: 72%. m.p.: 285°C (dec). <sup>1</sup>H NMR (DMSO-d<sub>6</sub>) δ: 0.8-1.7 (m, 38H), 1.75-1.9 (m 2H), 2.1-2.3  
15 (m, 2H), 2.9-3.0 (m, 1H), 3.8-3.9 (m, 2H), 4.5-4.6 (m, 1H), 4.56 (s, 1H), 4.69 (s, 1H), 8.30 (br, 3H). MS (ESI<sup>+</sup>): 516.1 (M+1). FT-IR (cm<sup>-1</sup>): 2947, 2868, 1740. Anal. Calcd for C<sub>32</sub>H<sub>54</sub>ClNO<sub>4</sub>: C, 69.60; H, 9.86; N, 2.54; Found: C, 65.76; H, 9.62; N, 2.63.

20 Antimelanoma Activity Assays

The relative efficacies of the prodrugs of betulinic acid and betulinic acid derivatives versus cancer cells can be established by determining the concentrations at which each prodrug inhibits the  
25 cancer cell activity to a predefined extent and then comparing the results. Typically, the preferred determination is the concentration that inhibits 50% of the activity in a biochemical assay, i.e., the 50% inhibitory concentration or "IC<sub>50</sub>." IC<sub>50</sub> deter-  
30 minations can be accomplished using conventional techniques known in the art. In general, an IC<sub>50</sub> can

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be determined by measuring the activity of a given cell line in the presence of a range of concentrations of the compound under study. The experimentally obtained values of cell line activity then  
5 are plotted against the compound concentrations used. The concentration of the inhibitor that shows 50% cell line activity (as compared to the activity in the absence of any inhibitor) is taken as the  $IC_{50}$  value.

10 The  $IC_{50}$  values for the prodrugs of betulinic acid and betulinic acid derivatives were determined from concentration-response curves typically using concentrations ranging from 0.01 to 100  $\mu\text{g/mL}$ . The  $IC_{50}$  determinations can be accomplished  
15 by well-known methods in the art. An exemplary test method is as follows:

The cytotoxic potential of test compounds was determined with various cell lines as described previously in K. Likhitwitayawuid et al., *J. Nat. Prod.*, 56, pages 1468-1478 (1993). Briefly, various  
20 concentrations of test compounds (e.g., 0.1 to 100  $\mu\text{g/mL}$ , dissolved in 10 L of 10% DMSO) were transferred to 96 well plates, and 190 L aliquots of cell suspensions (e.g.,  $5 \times 10^4$  cells/ml) were added to each  
25 well. The plates then were incubated for 72 hours at 37°C (100% humidity with a 5%  $\text{CO}_2$  atmosphere in air), and 100 L of cold 20% aqueous trichloroacetic acid was added to the growth medium in each well to fix the cells. The cultures were incubated at 4°C  
30 for 30 minutes, washed, air dried, stained with sulforhodamine B solution, and washed with 1% acetic acid. Finally, 200 L of 10 mM Tris base was added to each well and the optical densities were determined at 515 nm utilizing an ELISA plate reader. In  
35 each case, a zero day control was performed by

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adding an equivalent number of cells to several wells and incubating at 37°C for 30 minutes, and processing as described above. Optical density values obtained with the zero day control were subtracted, and cell survival, relative to control (solvent-treated) cultures, was calculated. Results were expressed as IC<sub>50</sub> values (i.e., concentration of test compound required to reduce cell number by 50%).

The derivatized betulinic acid prodrugs were tested against melanoma (Mel 2), and the *in vitro* results are summarized in the following table.

Compound	IC <sub>50</sub> (µg/mL)	
	Mel 2 (10% DMSO) <sup>a</sup>	Mel 2 (Media) <sup>b</sup>
27a	>20	>20
27d	>20	>20
27c	>20	>20
27e	>20	>20
27f	>20	>20
4a	>20	9.6
4i	5.0	4.0
4j	3.0	1.9
4f	3.3	2.7
28a	1.6	2.8

<sup>a</sup> The test articles were dissolved in 10% DMSO.

<sup>b</sup> The test articles were dissolved in media.

To test the *in vivo* ability of betulinic acid to serve as an antineoplastic agent against malignant melanoma, a series of studies was performed with athymic (nude) mice injected subcutaneously with human melanoma cells (MEL-2). The ini-



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tial study investigated the activity of betulinic acid against unestablished tumors. Treatment with betulinic acid began on day 1, i.e., 24 hours, following tumor cell injection. At doses of 50, 250, and 500 mg/kg (milligram per kilogram) body weight, betulinic acid demonstrated effective inhibition of tumor growth with p values of 0.001 for each dose versus a control.

In particular, the data was derived from experiments wherein four week old athymic mice were injected subcutaneously in the right flank with  $3.0 \times 10^8$  UIISO MEL-2 cells. UIISO MEL-2 is a cell line derived from metastatic melanoma from human pleural fluid. Drug treatment was initiated on the day following tumor cell injection and continued every fourth day for a total of six doses. Four control animals received 0.5 ml intraperitoneal (IP) of PVP control solution, while treated animals (4 per group) received 50, 250 or 500 mg/kg/dose IP betulinic acid/PVP in deionized  $H_2O$ . Betulinic acid was coprecipitated with PVP to increase solubility and bioavailability. The mice were weighed, and the tumors measured with a micrometer every other day throughout the study. All animals were sacrificed and autopsied on day 33, when the mean tumor volume in the control animals was approximately one  $cm^3$ .

There was greater inhibition of tumor growth at the highest dose of betulinic acid versus the lowest dose ( $p = 0.04$ ). Toxicity was not associated with the betulinic acid treatment because toxicity is indicated by loss of body weight or other forms of acute toxicity. No weight loss was observed.

Next, *in vivo* testing of betulinic acid was performed on established melanomas. In this

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study, treatment was withheld until day 13, by which time a palpable tumor mass was present in all mice. Under these conditions betulinic acid successfully abrogated tumor growth ( $p = 0.0001$ ). Furthermore, tumor growth did not parallel that of the control (untreated) group even 14 days after the termination of treatment.

In particular, four-week-old athymic mice were injected with  $5 \times 10^8$  MEL-2 cells subcutaneously in the right flank. Four treatment groups of five mice each were studied. In one group, the mice received 250 mg/kg/dose of IP betulinic acid/PVP every third day for six total doses initiated the day following tumor cell injection. The control group received 0.5 ml IP saline. A DTIC treatment group received 4 mg/kg/dose IP DTIC every third day from day 13 to day 28 of the study. The betulinic acid treatment group received 250 mg/kg/dose IP betulinic acid/PVP every third day from day 13 to day 27. The control and DTIC-treated mice were sacrificed and autopsied on day 36 due to their large tumor burden. The remaining mice were sacrificed and autopsied on day 41.

The efficacy of betulinic acid also was compared to DTIC, which is clinically available for the treatment of metastatic melanoma. The dose of DTIC, which is limited by toxicity, was selected to be equivalent to that administered to human patients. Tumor growth in the betulinic acid-treated group was significantly less than that observed in the DTIC-treated animals ( $p=0.0001$ ). Compared to controls, DTIC produced a significant, but less pronounced, reduction in tumor growth, with a  $p$  value of 0.01. A fourth group in this study was treated with a schedule similar to that in the

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initial study. Under these conditions, betulinic acid, as demonstrated before, significantly inhibited tumor development ( $p=0.0001$ ) and caused a prolonged reduction in tumor growth of up to three  
5 weeks following treatment termination.

Betulinic acid also showed activity against MEL-1 cells. In particular, four-week-old athymic mice were injected subcutaneously in the right flank with  $5.0 \times 10^8$  UISO MEL-1 cells. Drug  
10 treatment was initiated on the day following tumor cell injection and continued every fourth day for a total of six doses. Four control animals received 0.5 ml intraperitoneal (IP) saline, while treated  
15 animals (4 per group) received 5, 50 or 250 mg/kg/-dose IP betulinic acid/PVP in dd H<sub>2</sub>O. The mice were weighed, and tumors were measured with a micrometer every third day throughout the study. Treated  
animals were sacrificed and autopsied on day 41, when the mean tumor volume in the control mice was  
20 approximately 0.5 cm<sup>3</sup>. The control mice then received six doses of 50 mg/kg every fourth day beginning day 41 and were sacrificed and autopsied on day 71.

The results with respect to MEL-1 cells  
25 were similar to the results with respect to MEL-2 cells. Betulinic acid therefore is active both against MEL-1 and MEL-2 cells. See, Pezzuto et al. U.S. Patent No. 5,962,527, incorporated herein by  
reference, for additional information with respect  
30 to the *in vivo* activity of betulinic acid and derivatives against melanoma, and for *in vivo* activity against other forms of cancer.

Taking into account a unique *in vitro* cytotoxicity profile, a significant *in vivo* activ-  
35 ity, and mode of action, betulinic acid is an ex-

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ceptionally attractive compound for treating human melanoma. Betulinic acid also is relatively innocuous toxicitywise, as evidenced by repeatedly administering 500 mg/kg doses of betulinic acid without causing acute signs of toxicity or a decrease in body weight. Betulinic acid was previously found to be inactive in a Hippocratic screen at 200 and 400 mg/kg doses.

In particular, betulinic acid derivatives have been synthesized and evaluated biologically to illustrate that betulinic acid and betulinic acid derivatives possess selective antitumor activity against human melanoma cells lines *in vitro*. It has been demonstrated that modifying the parent structure of betulinic acid and betulinic acid derivatives provide numerous prodrugs that can be easily formulated and administered to an individual, which release betulinic acid or a betulinic acid derivative and used to prevent or inhibit malignant tumor growth, especially with respect to human melanoma. The antitumor activity of betulinic acid and betulinic acid derivatives is important therapeutically because these compounds exhibit a high activity against melanomas, but the compounds also possess a low water solubility. The low water solubility of betulinic acid and derivatives, however, can be overcome by providing an appropriate prodrug of betulinic acid or a betulinic acid derivative.

The above synthetic schemes show that modifying the betulinic acid and betulinic acid derivatives can provide prodrugs capable of releasing betulinic acid or a betulinic acid derivative *in vivo*. The prodrugs, therefore, can be used as potent antitumor drugs against melanoma and other cancers. The preparation of a prodrug overcomes the

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low solubility of betulinic acid and betulinic acid derivatives in water.

It is envisioned, therefore, that prodrugs of betulinic acid and betulinic acid derivatives are useful in the treatment of various cancers, for example, melanoma, a squamous tumor, a breast cancer, a colon cancer, a sarcoma, a human oral epidermoid carcinoma, a prostate cancer, a lung cancer, a glioma, or a neuroblastoma. Thus, the present invention concerns the use of prodrugs of betulinic acid and betulinic acid derivatives, or a pharmaceutical composition containing such an entity, for the manufacture of a medicament for the curative or prophylactic treatment of a cancer in a mammal, including humans.

The term "treatment" includes preventing, lowering, stopping, or reversing the progression or severity of the condition or symptoms being treated. As such, the term "treatment" includes both medical therapeutic and/or prophylactic administration, as appropriate.

It also is understood that "a prodrug of betulinic acid or a betulinic acid derivative" can be administered as the neat compound, or as a pharmaceutical composition containing such an entity.

In a further aspect, the present invention provides a method of treating a cancer in a human or nonhuman animal body which comprises administering to said body a therapeutically effective amount of a prodrug of betulinic acid or a betulinic acid derivative.

The prodrugs of the invention can be administered by any suitable route, for example by oral, buccal, inhalation, sublingual, rectal, vaginal, transurethral, nasal, topical, percutaneous,

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i.e., transdermal, or parenteral (including intravenous, intramuscular, subcutaneous, and intracoronary) administration. Parenteral administration can be accomplished using a needle and syringe, or  
5 using a high pressure technique, like POWDERJECT™.

Compounds and pharmaceutical compositions suitable for use in the present invention include those wherein the prodrug is administered in an effective amount to achieve its intended purpose.  
10 More specifically, a "therapeutically effective amount" means an amount effective to treat or to prevent development of, or to alleviate the existing symptoms of, the subject being treated. Determination of the effective amounts is well within the  
15 capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

A "therapeutically effective dose" refers to that amount of the prodrug that results in achieving the desired effect. Toxicity and therapeutic efficacy of the prodrugs can be determined by  
20 standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50%  
25 of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, which is expressed as the ratio between LD<sub>50</sub> and ED<sub>50</sub>. Compounds which exhibit high therapeutic indices are preferred. The data obtained can be used in formulating a dosage range for use in humans. The dosage of such compounds preferably lies within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The dosage can vary within this range depending upon the dosage form  
30 employed, and the route of administration utilized.  
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The exact formulation, route of administration, and dosage can be chosen by the individual physician in view of the particular disease being treated and the patient's condition. Dosage amount  
5 and interval can be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the therapeutic effects.

The amount of prodrug administered is dependent on the subject being treated, on the subject's weight, the severity of the affliction, the  
10 manner of administration, and the judgment of the prescribing physician.

Specifically, for administration to a human in the curative or prophylactic treatment of  
15 the conditions and disorders identified above, dosage of the prodrugs of the present invention generally are about 0.5 to about 1000 mg daily for an average adult patient (70 kg). Thus, for a typical adult patient, individual tablets or capsules contain  
20 0.2 to 500 mg of prodrug, in a suitable pharmaceutically acceptable vehicle or carrier, for administration in single or multiple doses, once or several times per day. Dosages for intravenous, buccal, or sublingual administration typically are  
25 0.1 to 500 mg per single dose as required. In practice, the physician determines the actual dosing regimen which is most suitable for an individual patient, and the dosage varies with the age, weight, and response of the particular patient. The above  
30 dosages are exemplary of the average case, but there can be individual instances in which higher or lower dosages are merited, and such are within the scope of this invention.

For human use, a prodrug of the present  
35 invention can be administered alone, but generally

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is administered in admixture with a pharmaceutical carrier selected with regard to the intended route of administration and standard pharmaceutical practice. Pharmaceutical compositions for use in accordance with the present invention thus can be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the prodrugs into preparations which can be used pharmaceutically.

These pharmaceutical compositions can be manufactured in a conventional manner, e.g., by conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. Proper formulation is dependent upon the route of administration chosen. When a therapeutically effective amount of a prodrug of the present invention is administered orally, the composition typically is in the form of a tablet, capsule, powder, solution, or elixir. When administered in tablet form, the composition can additionally contain a solid carrier, such as a gelatin or an adjuvant. The tablet, capsule, and powder contain about 5 to about 95% prodrug of the present invention, and preferably from about 25 to about 90% compound of the present invention. When administered in liquid form, a liquid carrier such as water, petroleum, or oils of animal or plant origin can be added. The liquid form of the composition can further contain physiological saline solution, dextrose or other saccharide solutions, or glycols. When administered in liquid form, the composition contains about 0.5 to about 90% by weight of a prodrug of the present



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invention, and preferably about 1 to about 50% of a compound of the present invention.

When a therapeutically effective amount of a prodrug of the present invention is administered  
5 by intravenous, cutaneous, or subcutaneous injection, the composition is in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable solutions, having due regard to pH, isotonicity, stability,  
10 ity, and the like, is within the skill in the art. A preferred composition for intravenous, cutaneous, or subcutaneous injection typically contains, in addition to a compound of the present invention, an isotonic vehicle.

15 For oral administration, the compounds can be formulated readily by combining a prodrug of the present invention with pharmaceutically acceptable carriers well known in the art. Such carriers enable the present compounds to be formulated as  
20 tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained by adding a compound of formula (I) with a solid excipient, optionally grinding a resulting mixture, and  
25 processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients include, for example, fillers and cellulose preparations. If  
30 desired, disintegrating agents can be added.

For administration by inhalation, compounds of the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the  
35 use of a suitable propellant. In the case of a

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pressurized aerosol, the dosage unit can be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin, for use in an inhaler or insufflator can be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The prodrugs can be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, e.g., in ampules or in multidose containers, with an added preservative. The compositions can take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing, and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the prodrugs in water-soluble form. Additionally, suspensions of the prodrugs can be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils or synthetic fatty acid esters. Aqueous injection suspensions can contain substances which increase the viscosity of the suspension. Optionally, the suspension also can contain suitable stabilizers or agents that increase the solubility of the compounds and allow for the preparation of highly concentrated solutions. Alternatively, a present composition can be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

Prodrugs of the present invention also can be formulated in rectal compositions, such as suppositories or retention enemas, e.g., containing

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conventional suppository bases. In addition to the formulations described previously, the prodrugs also can be formulated as a depot preparation. Such long-acting formulations can be administered by  
5 implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds can be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion  
10 exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

In particular, a prodrug of the present invention can be administered orally, buccally, or sublingually in the form of tablets containing  
15 excipients, such as starch or lactose, or in capsules or ovules, either alone or in admixture with excipients, or in the form of elixirs or suspensions containing flavoring or coloring agents. Such liquid preparations can be prepared with pharmaceut-  
20 ically acceptable additives, such as suspending agents. A prodrug also can be injected parenterally, for example, intravenously, intramuscularly, subcutaneously, or intracoronarily. For parenteral administration, the prodrug is best used in the form  
25 of a sterile aqueous solution which can contain other substances, for example, salts, or monosaccharides, such as mannitol or glucose, to make the solution isotonic with blood.

For veterinary use, a prodrug of the present invention or a nontoxic salt thereof, is admin-  
30 istered as a suitably acceptable formulation in accordance with normal veterinary practice. The veterinarian can readily determine the dosing regimen and route of administration that is most  
35 appropriate for a particular animal.

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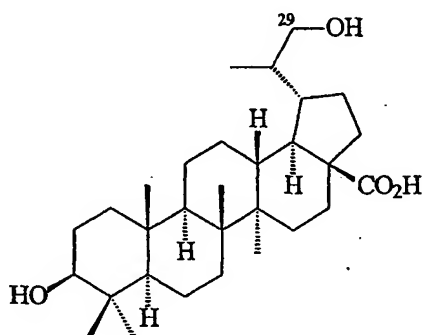
Thus, the invention provides in a further aspect a pharmaceutical composition comprising a prodrug of the present invention, together with a pharmaceutically acceptable diluent or carrier therefor. There is further provided by the present invention a process of preparing a pharmaceutical composition comprising mixing a prodrug of the present invention, together with a pharmaceutically acceptable diluent or carrier therefor.

10 In a particular embodiment, the invention includes a pharmaceutical composition for the curative or prophylactic treatment of a cancer in a mammal, including humans, comprising prodrug of the present invention, together with a pharmaceutically acceptable diluent or carrier.

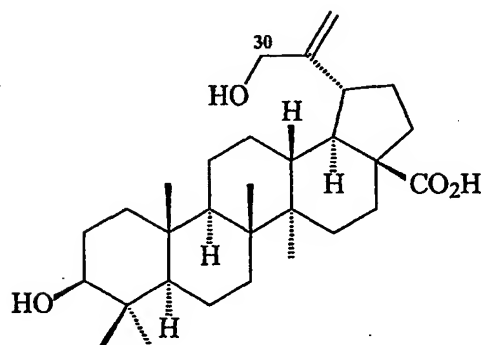
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## WHAT IS CLAIMED IS:

1. A composition for treating a tumor growth comprising: (a) therapeutically effective amount of a compound having a formula



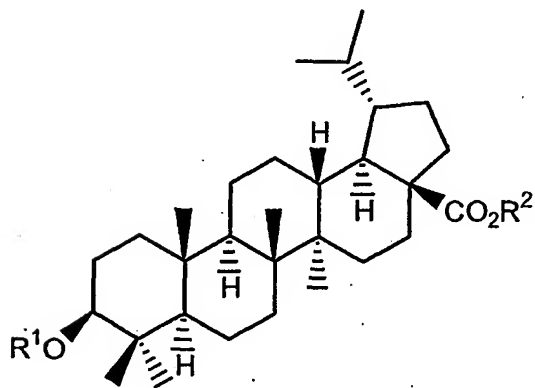
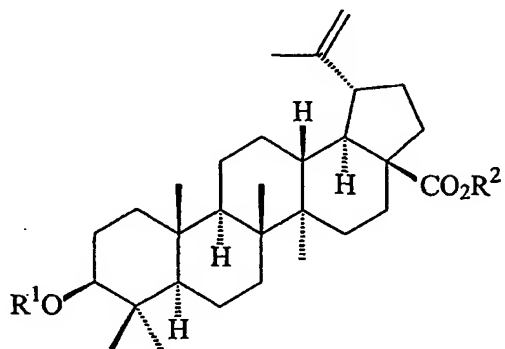
or



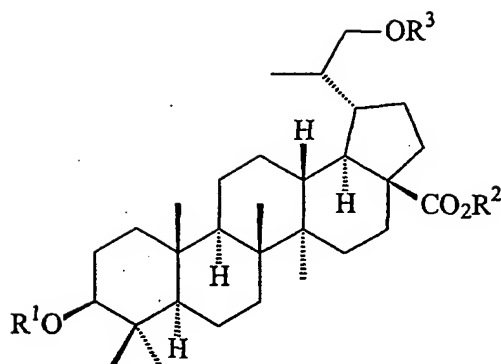
and (b) an optional carrier.

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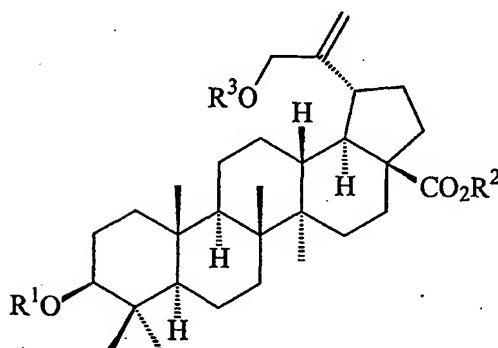
2. A composition for treating a tumor growth comprising: (a) a prodrug of betulinic acid or a derivative thereof, selected from the group consisting of



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, and



wherein R<sup>1</sup> and R<sup>3</sup>, independently, are selected from the group consisting of hydrogen, CO(C<sub>1</sub>-C<sub>6</sub>alkyl)NR<sup>4</sup>R<sup>5</sup>, CO(C<sub>1-3</sub>alkyl)CO<sub>2</sub>R<sup>4</sup>, COCH(C<sub>6</sub>H<sub>5</sub>)NR<sup>4</sup>R<sup>5</sup>, CO(C<sub>1</sub>-C<sub>6</sub>alkyl), CO(C<sub>1</sub>-C<sub>6</sub>alkyl)CO<sub>2</sub>R<sup>4</sup>, CO(C<sub>1-6</sub>alkyl)O-(CH<sub>2</sub>CH<sub>2</sub>O)<sub>n</sub>C<sub>1-3</sub>alkyl, CH<sub>2</sub>OCO<sub>2</sub>C<sub>1-6</sub>alkyl, CH<sub>2</sub>OCOC<sub>1-6</sub>alkyl, PO(OH)<sub>2</sub>, and SO<sub>3</sub>H,

$R^2$  is selected from the group consisting of hydrogen,  $C_1$ - $C_6$ alkyl,  $CH_2C_6H_5$ ,  $C_1$ - $C_6$ alkyl $NR^4R^5$ ,  $CH_2OCOC_1$ - $C_6$ alkyl,  $PO(OH)_2$ ,  $SO_3H$ ,  $CH(C_6H_5)NR^4R^5$ ,  $(C_1$ - $C_6$ alkyl) $CO_2R^4$ , and  $(C_1$ - $C_6$ alkyl) $O(CH_2CH_2O)_n C_{1-3}$ alkyl,

- 71 -

R<sup>4</sup> and R<sup>5</sup>, independently, are selected from the group consisting of hydrogen, C<sub>1</sub>-C<sub>6</sub>alkyl, CO(C<sub>1</sub>-C<sub>6</sub>alkyl), and aryl, or R<sup>4</sup> and R<sup>5</sup> can be taken together to form a 5 to 7 membered ring,

and n is 1 to 10;

and pharmaceutically acceptable salts thereof,

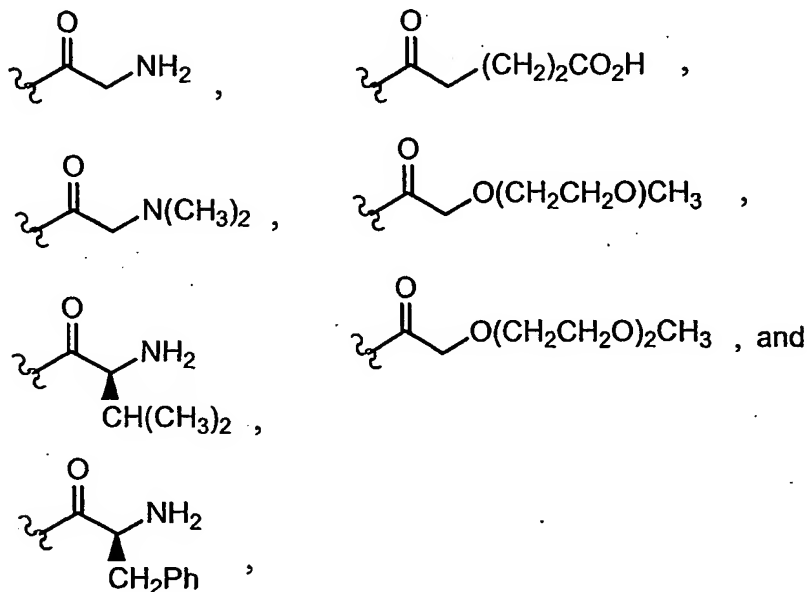
and (b) an optional carrier.



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3. The composition of claim 2 wherein at least one of  $R^1$ ,  $R^2$ , and  $R^3$  is hydrogen, and at least one of  $R^1$ ,  $R^2$ , and  $R^3$  is different from hydrogen.

4. The composition of claim 2 wherein  $R^1$ ,  $R^2$ , and  $R^3$  are selected from the group consisting of



wherein Ph is  $\text{C}_6\text{H}_5$ .

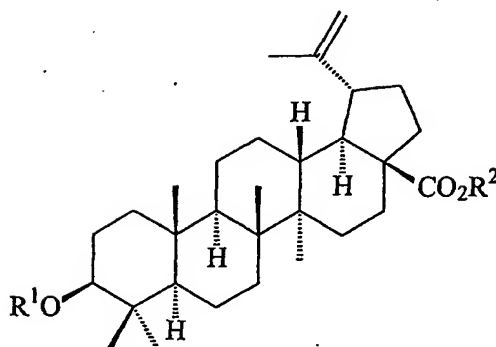
5. The composition of claim 2 wherein at least one of  $R^1$ ,  $R^2$ , and  $R^3$  is  $\text{PO}(\text{OH})_2$ , and the remaining  $R^1$ ,  $R^2$ , and  $R^3$  are hydrogen.

6. The composition of claim 2 wherein at least one of  $R^1$ ,  $R^2$ , and  $R^3$  is  $\text{CH}_2\text{OCOC}(\text{CH}_3)_3$ .

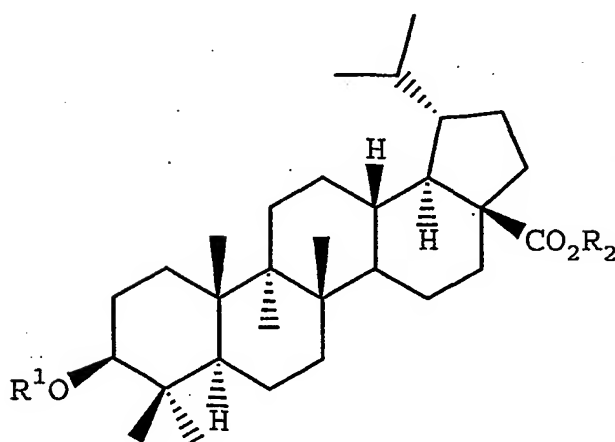
7. The composition of claim 2 wherein at least one of  $R^1$ ,  $R^2$ , and  $R^3$  is  $\text{SO}_3\text{H}$  and the remaining  $R^1$ ,  $R^2$ , and  $R^3$  are hydrogen.

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8. A compound having a structure:



or



wherein  $R^1$  is selected from the group consisting of hydrogen,  $CO(C_1-C_6\text{alkyl})NR^4R^5$ ,  $CO(C_{1-3}\text{-alkyl})CO_2R^4$ ,  $COCH(C_6H_5)NR^4R^5$ ,  $CO(C_1-C_6\text{alkyl})$ ,  $CO(C_1-C_6\text{alkyl})CO_2R^4$ ,  $CO(C_{1-6}\text{alkyl})O(CH_2CH_2O)_nC_{1-3}\text{alkyl}$ ,  $CH_2OCO_2C_{1-6}\text{alkyl}$ ,  $CH_2OCOC_{1-6}\text{alkyl}$ ,  $PO(OH)_2$ , and  $SO_3H$ .

$R^2$  is selected from the group consisting of hydrogen,  $C_1-C_6\text{alkyl}$ ,  $CH_2C_6H_5$ ,  $C_1-C_6\text{alkyl}NR^4R^5$ ,  $CH_2OCOC_{1-6}\text{alkyl}$ .

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C<sub>6</sub>alkyl, PO(OH)<sub>2</sub>, SO<sub>3</sub>H, CH(C<sub>6</sub>H<sub>5</sub>)NR<sup>4</sup>R<sup>5</sup>, (C<sub>1</sub>-C<sub>6</sub>alkyl)CO<sub>2</sub>R<sup>4</sup>,  
and (C<sub>1</sub>-C<sub>6</sub>alkyl)O(CH<sub>2</sub>CH<sub>2</sub>O)<sub>n</sub>C<sub>1-3</sub>alkyl,

R<sup>4</sup> and R<sup>5</sup>, independently, are selected from  
the group consisting of hydrogen, C<sub>1</sub>-C<sub>6</sub>alkyl, CO(C<sub>1</sub>-  
C<sub>6</sub>alkyl), and aryl, or R<sup>4</sup> and R<sup>5</sup> can be taken together  
to form a 5 to 7 membered ring,

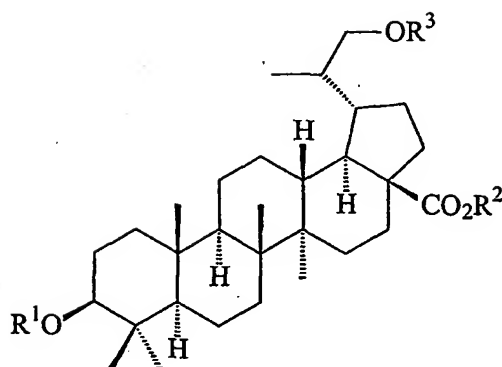
and n is 1 to 10;

with proviso that at least one of R<sup>1</sup> and R<sup>2</sup>  
is different from H;

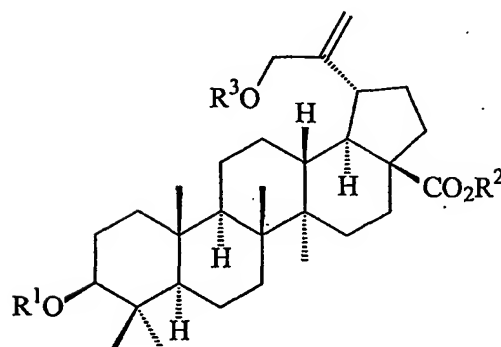
and pharmaceutically acceptable salts  
thereof.

- 75 -

9. A compound having a structure:



or



wherein  $R^1$  and  $R^3$ , independently, are selected from the group consisting of hydrogen,  $CO(C_1-C_6\text{alkyl})NR^4R^5$ ,  $CO(C_{1-3}\text{alkyl})CO_2R^4$ ,  $COCH(C_6H_5)NR^4R^5$ ,  $CO(C_1-C_6\text{alkyl})$ ,  $CO(C_1-C_6\text{alkyl})CO_2R^4$ ,  $CO(C_{1-6}\text{alkyl})-O(CH_2CH_2O)_nC_{1-3}\text{alkyl}$ ,  $CH_2OCO_2C_{1-6}\text{alkyl}$ ,  $CH_2OCOC_{1-6}\text{alkyl}$ ,  $PO(OH)_2$ , and  $SO_3H$ ,

$R^2$  is selected from the group consisting of hydrogen,  $C_1-C_6\text{alkyl}$ ,  $CH_2C_6H_5$ ,  $C_1-C_6\text{alkyl}NR^4R^5$ ,  $CH_2OCOC_{1-6}\text{alkyl}$ ,  $PO(OH)_2$ ,  $SO_3H$ ,  $CH(C_6H_5)NR^4R^5$ ,  $(C_1-C_6\text{alkyl})CO_2R^4$ , and  $(C_1-C_6\text{alkyl})O(CH_2CH_2O)_nC_{1-3}\text{alkyl}$ ,

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R<sup>4</sup> and R<sup>5</sup>, independently, are selected from the group consisting of hydrogen, C<sub>1</sub>-C<sub>6</sub>alkyl, CO(C<sub>1</sub>-C<sub>6</sub>alkyl), and aryl, or R<sup>4</sup> and R<sup>5</sup> can be taken together to form a 5 to 7 membered ring,

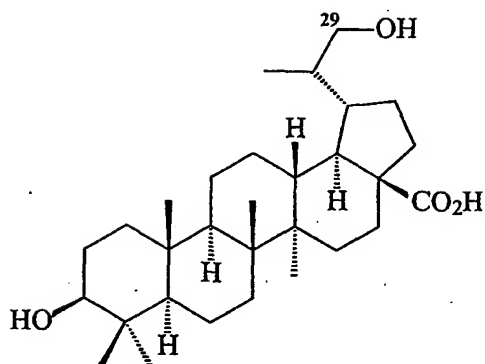
and n is 1 to 10;

and pharmaceutically acceptable salts

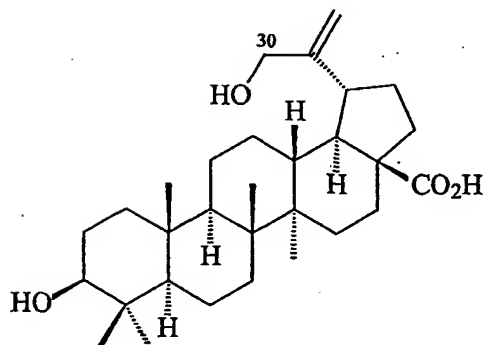
thereof.

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10. A compound of claim 9 wherein the compound has the structure:



OR



11. A compound as described herein, and identified as compound 2, 3, 4a through 4j, 5a through 5g, 6a through 6g, 7, 8, 9, 10a, 10b, 11a through 11g, 12a through 12g, 13, 14a through 14c, 15a through 15c, 22, 23, 26, 27a through 27i, 28a through 28i, and salts thereof.

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12. A method of treating a cancer sensitive to betulinic acid or a betulinic acid derivative comprising administering to an individual in need thereof a therapeutically effective amount of a compound of claim 11.

13. The method of claim 12 wherein the cancer is selected from the group consisting of a melanoma, a squamous tumor, a breast cancer, a colon cancer, a sarcoma, a human oral epidermal carcinoma, a hormone-dependent breast cancer, a prostate cancer, a lung cancer, a glioma, a melanoma, and a neuroblastoma.

14. A method of treating a cancer sensitive to betulinic acid or a betulinic acid derivative comprising administering to an individual in need thereof a therapeutically effective amount of a prodrug of betulinic acid or a prodrug of a betulinic acid derivative.

15. The method of claim 14 wherein the cancer is selected from the group consisting of a melanoma, a squamous tumor, a breast cancer, a colon cancer, a sarcoma, a human oral epidermal carcinoma, a hormone-dependent breast cancer, a prostate cancer, a lung cancer, a glioma, a melanoma, and a neuroblastoma.

16. The method of claim 14 wherein the prodrug is administered topically, intravenously, or intraperitoneally.

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17. A method of treating HIV comprising administering to an individual in need thereof a therapeutically effective amount of a compound of claim 11.

18. A method of treating HIV comprising administering to an individual in need thereof a therapeutically effective amount of a prodrug of betulinic acid or a prodrug of a betulinic acid derivative.

19. The method of claim 18 wherein the prodrug is administered topically, intravenously, or intraperitoneally.

20. A pharmaceutical composition comprising a compound of claim 11 and a pharmaceutically acceptable carrier.

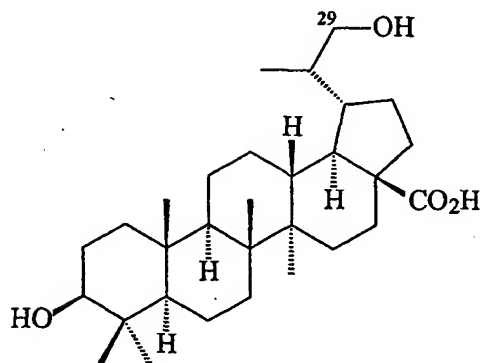
21. A method of treating a cancer sensitive to betulinic acid or a betulinic acid derivative comprising administering to an individual in need thereof a therapeutically effective amount of a composition comprising a compound of claim 11 and a carrier.

22. A method of treating HIV comprising administering to an individual in need thereof a therapeutically effective amount of a composition comprising a compound of claim 11 and a carrier.



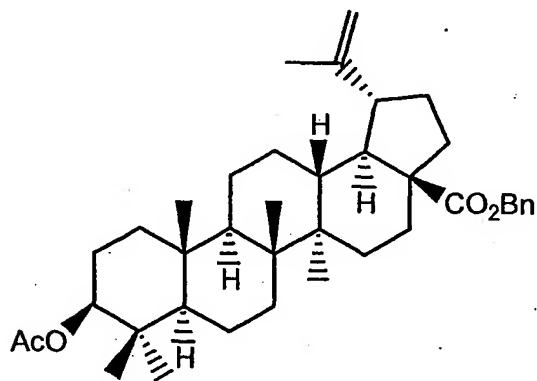
- 80 -

23. A process of preparing a compound having a structure



comprising the steps of:

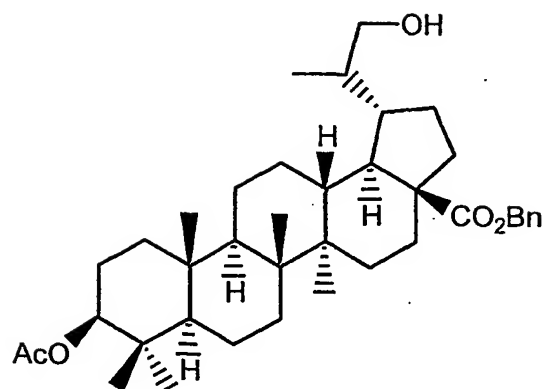
(a) hydroborating a protected betulinic acid having a structure



with  $\text{BH}_3$ -dimethyl sulfide;

(b) oxidizing the reaction product of step (a) under basic conditions to yield a C-29 alcohol having a structure

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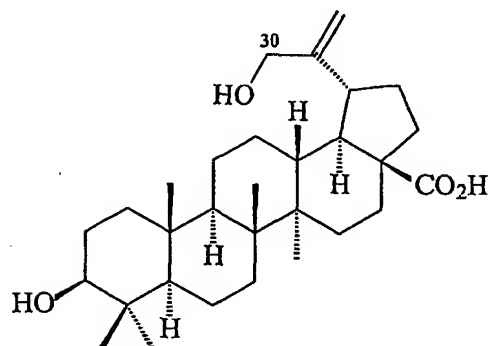


(c) hydrolyzing the C-3 acetate group of the product of step (b);

then (d) a catalytic hydrogenolysis of the C-28 benzyl ester group of the product of step (c).

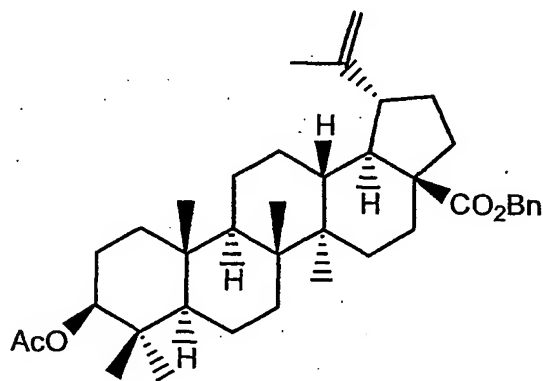
- 82 -

24. A process of preparing a compound having a structure



comprising the steps of:

(a) oxidizing a compound having a structure



using catalytic selenium dioxide and *t*-butyl hydroperoxide;

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(b) hydrolyzing a C-3 acetate group of the product of step (a);

and (c) a catalytic hydrogenolysis of the C-28 benzyl ester group of the product of step (b).

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 01/25581

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07J63/00 A61K31/56 A61P35/00 A61P31/18

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07J A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, BEILSTEIN Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>FUJIOKA T ET AL: "ANTI-AIDS AGENTS, 11. BETULINIC ACID AND PLATANIC ACID AS ANTI-HIV PRINCIPLES FROM SYZIGIUM CLAVIFLORUM, AND THE ANTI-HIV ACTIVITY OF STRUCTURALLY RELATED TRITERPENOIDs" JOURNAL OF NATURAL PRODUCTS, XX, XX, vol. 57, no. 2, 1 February 1994 (1994-02-01), pages 243-247, XP000612854 ISSN: 0163-3864 page 244; examples 9,10 page 245; table 1</p> <p style="text-align: center;">-/--</p>	1-11, 17-22

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

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- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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Date of the actual completion of the international search

6 December 2001

Date of mailing of the international search report

21/12/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Watchorn, P

## INTERNATIONAL SEARCH REPORT

In International Application No

PCT/US 01/25581

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUN, I-CHEN ET AL: "Anti- AIDS Agents. 34. Synthesis and Structure-Activity Relationships of Betulin Derivatives as Anti- HIV Agents" J. MED. CHEM. (1998), 41(23), 4648-4657 , XP002184894 page 4649, column 1, paragraph 1; examples 2-5 page 4650; examples 26-29	1-11, 17-22
X	YASUKAWA K ET AL: "SOME LUPANE-TYPE TRITERPENES INHIBIT TUMOR PROMOTION BY 12-O-TETRADECANOYLPHORBOL-13-ACETATE IN TWO-STAGE CARCINOGENESIS IN MOUSE SKIN" PHYTOMEDICINE, GUSTAV FISCHER VERLAG, STUTTGART, DE, vol. 4, 1995, pages 309-313, XP000610925 ISSN: 0944-7113 page 311; table 1	1-16
Y		12-16
X	WO 93 10142 A (RHONE POULENC RORER SA) 27 May 1993 (1993-05-27) page 91, line 22-29; examples 9,11,12	1-11, 17-22
X	WO 94 26725 A (RHONE POULENC RORER SA ;DEREU NORBERT (FR); EVERS MICHEL (BE); POU) 24 November 1994 (1994-11-24)	9
Y	page 46, line 19-28; example 3	1-11, 17-22
X	DATABASE CA 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; MACIAS, FRANCISCO A. ET AL: "'Natural products as allelochemicals".5. Bioactive steroids and triterpenes from Melilotus messanensis and their allelopathic potential" retrieved from STN Database accession no. 127:231877 XP002184898 abstract & J. CHEM. ECOL. (1997), 23(7), 1781-1803	9,10

## INTERNATIONAL SEARCH REPORT

Int'l Patent Application No  
PCT/US 01/25581

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE CA 'Online!            CHEMICAL ABSTRACTS SERVICE, COLUMBUS,            OHIO, US;            PRADHAN, BHIM PRASAD ET AL: "Oxidation of            triterpenoids: Part XVII. Oxidation of            isopropenyl double bond of lupane skeleton            with m-chloroperbenzoic acid in a molar            proportion"            retrieved from STN            Database accession no. 123:9720            XP002184899            abstract            &amp; INDIAN J. CHEM., SECT. B: ORG. CHEM.            INCL. MED. CHEM. (1995), 34B(6), 540-42 ,</p>	9
X	<p>DATABASE CA 'Online!            CHEMICAL ABSTRACTS SERVICE, COLUMBUS,            OHIO, US;            DINA, B. ET AL: "Reactions on naturally            occurring triterpene: part 1"            retrieved from STN            Database accession no. 123:169934            XP002184900            abstract            &amp; INDIAN J. CHEM., SECT. B: ORG. CHEM.            INCL. MED. CHEM. (1995), 34B(7), 624-8 ,</p>	9
X	<p>DATABASE CA 'Online!            CHEMICAL ABSTRACTS SERVICE, COLUMBUS,            OHIO, US;            PATRA, AMARENDRA ET AL: "Studies on            triterpenoids: treatment of            3-acetylbetulinic acid with            m-chloroperbenzoic acid and sulfuric acid"            retrieved from STN            Database accession no. 111:78441            XP002184901            abstract            &amp; INDIAN J. CHEM., SECT. B (1988), 27B(2),            170-2 ,</p> <p style="text-align: center;">-/-</p>	9

## INTERNATIONAL SEARCH REPORT

In — tional Application No

PCT/US 01/25581

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>DATABASE CA 'Online!            CHEMICAL ABSTRACTS SERVICE, COLUMBUS,            OHIO, US;            TANAKA, REIKO ET AL: "Bioactive Steroids            from the Whole Herb of Euphorbia            chamaesyce"            retrieved from STN            Database accession no. 132:134799            XP002184902            abstract            &amp; J. NAT. PROD. (2000), 63(1), 99-103 ,</p>	1-11, 17-22
Y	<p>DATABASE CA 'Online!            CHEMICAL ABSTRACTS SERVICE, COLUMBUS,            OHIO, US;            KUO, YAO-HAUR ET AL: "Cytotoxic            constituents from the fruit of Diospyros            ferrea"            retrieved from STN            Database accession no. 128:274929            XP002184903            abstract            &amp; CHIN. PHARM. J. (TAIPEI) (1997), 49(4),            207-216 ,</p>	1-11, 17-22
Y	<p>EVERS, MICHEL ET AL: "Betulinic Acid            Derivatives: A New Class of Human            Immunodeficiency Virus Type 1 Specific            Inhibitors with a New Mode of Action"            J. MED. CHEM. (1996), 39(5), 1056-68 ,            XP002184895            page 1061; example 17B; table 1</p>	1-11, 17-22
X	<p>MAYAUX J-F ET AL: "TRITERPENE DERIVATIVES            THAT BLOCK ENTRY OF HUMAN IMMUNODEFICIENCY            VIRUS TYPE 1 INTO CELLS"            PROCEEDINGS OF THE NATIONAL ACADEMY OF            SCIENCES OF USA, NATIONAL ACADEMY OF            SCIENCE. WASHINGTON, US,            vol. 91, no. 9, 1 April 1994 (1994-04-01),            pages 3564-3568, XP000612123            ISSN: 0027-8424            page 3565; figure 1            page 3566; figure 2</p>	1-11, 17-22



## INTERNATIONAL SEARCH REPORT

h onal Application No

101/JS 01/25581

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>DATABASE CA 'Online!            CHEMICAL ABSTRACTS SERVICE, COLUMBUS,            OHIO, US;            FANG, SHENG DING ET AL: "The chemistry of            toxic principles from Maytenus nemerosa"            retrieved from STN            Database accession no. 101:51677            XP002184904            abstract            &amp; PHYTOCHEMISTRY (1984), 23(3), 631-3 ,</p>	1-16
X	<p>MACIAS F A ET AL: "Bioactive polar            triterpenoids from melilotus messanensis"            PHYTOCHEMISTRY, PERGAMON PRESS, GB,            vol. 49, no. 3, October 1998 (1998-10),            pages 709-717, XP004290188            ISSN: 0031-9422            page 712; example 1</p>	9,10
X	<p>EP 0 943 620 A (DABUR RESEARCH FOUNDATION)            22 September 1999 (1999-09-22)            page 9-10; example 15</p>	1-16
X	<p>HASHIMOTO F ET AL: "Anti-AIDS Agents.            XXVII. Synthesis and Anti-HIV Activity of            Betulinic Acid and Dihydrobetulinic Acid            Derivatives"            BIOORGANIC &amp; MEDICINAL CHEMISTRY, ELSEVIER            SCIENCE LTD, GB,            vol. 5, no. 12, 1997, pages 2133-2143,            XP002102686            ISSN: 0968-0896            page 2134; figure 1; examples            2-7,10-15,17,19,20,22,23            page 2136; table 1            page 2137; tables 2,3            page 2138; table 4</p>	1-11, 17-22
X	<p>MA, CHAOMEI ET AL: "Inhibitory effects of            constituents from Cynomorium songaricum            and related triterpene derivatives on            HIV-1 protease"            CHEM. PHARM. BULL. (1999), 47(2), 141-145            ' XP002184896            page 143; examples 37-41; table 2</p>	1-8,11, 17-22

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## INTERNATIONAL SEARCH REPORT

h — International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIM D S H L ET AL: "Synthesis of betulinic acid derivatives with activity against human melanoma". BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, OXFORD, GB, vol. 8, no. 13, 7 July 1998 (1998-07-07), pages 1707-1712, XP004137114 ISSN: 0960-894X page 1711; table 1	1-16
X	NODA Y ET AL: "Enhanced Cytotoxicity of Some Triterpenes toward Leukemia L1210 Cells Cultured in Low pH Media: Possibility of a New Mode of Cell Killing" CHEMICAL AND PHARMACEUTICAL BULLETIN, PHARMACEUTICAL SOCIETY OF JAPAN. TOKYO, JP, vol. 45, no. 10, 1997, pages 1665-1670, XP002082673 ISSN: 0009-2363 page 1667; table 1	1-16
X	KITAJIMA, JUNICHI ET AL: "Two new triterpenoid sulfates from the leaves of Schefflera octophylla" CHEM. PHARM. BULL. (1990), 38(3), 714-16 , XP002184897 page 714, chart 1, compounds 4 and 5	2,3,7,8, 11
X	KASHIWADA ET AL: "Betulinic Acid and Dihydrobetulinic Acid Derivatives as Potent Anti-HIV Agents" JOURNAL OF MEDICINAL CHEMISTRY, AMERICAN CHEMICAL, vol. 39, no. 5, 1996, pages 1016-1017, XP002078035 page 1016, column 2; examples 2-5,7-10 page 1017; table 1	1-11, 17-22
P,X	WO 00 59492 A (FARISS MARC ;SMITH J DOYLE (US); UNIV WASHINGTON (US); UNIV VIRGIN) 12 October 2000 (2000-10-12) page 3 page 4, paragraph 3 page 6, paragraph 3 page 7, paragraph 1 example 7	1-16

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 8,14-16,18-19 (in part)

Claim 14 and 15, 16 dependent thereon, specify the use of a "betulinic acid derivative" or its "prodrug" in the treatment of cancer. Claim 18 and claim 19 dependent thereon claim the use of the same compounds in the treatment of HIV.

These claims relate to the use of an extremely large number of possible compounds in the above medical methods, that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Furthermore, the above term "betulinic acid derivative" does not have any well recognised meaning in this technical area and as such the scope of the above claims is unclear to an extent that it is not possible to conduct a meaningful search thereon.

Consequently, the search has been carried out for those parts of the application which do appear to be clear (and/or concise), namely the above mentioned uses of the compounds as defined in composition claims 1-7 and compound claims 8-11.

It should further be noted, that with regard to the search on the compounds of claim 8, the initial phase of the search revealed a very large number of documents relevant to the issue of novelty. So many documents were retrieved that it is impossible to determine which parts of the claim(s) may be said to define subject-matter for which protection might legitimately be sought (Article 6 PCT). For these reasons, a meaningful search over the whole breadth of the claim(s) is impossible. Consequently, the search has been restricted to the preferred compounds specified in claim 11 and embraced by the scope of claim 8. In any case claim 11 in as far as it specifies compounds falling within the scope of claim 8 still lacks novelty.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

In — tional Application No

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Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9310142	A	27-05-1993	FR 2683531 A1	14-05-1993
			CA 2121692 A1	27-05-1993
			EP 0542622 A1	19-05-1993
			EP 0613483 A1	07-09-1994
			FI 942181 A	11-05-1994
			WO 9310142 A1	27-05-1993
			HU 66959 A2	30-01-1995
			JP 7501068 T	02-02-1995
			MX 9206503 A1	01-05-1993
			NO 941666 A	05-05-1994
			US 5468888 A	21-11-1995
WO 9426725	A	24-11-1994	FR 2705097 A1	18-11-1994
			AU 6724494 A	12-12-1994
			CA 2162703 A1	24-11-1994
			EP 0698016 A1	28-02-1996
			WO 9426725 A1	24-11-1994
			JP 8509969 T	22-10-1996
			ZA 9403202 A	16-01-1995
EP 0943620	A	22-09-1999	EP 0943620 A2	22-09-1999
			US 6228850 B1	08-05-2001
			US 6214814 B1	10-04-2001
WO 0059492	A	12-10-2000	AU 4331700 A	23-10-2000
			WO 0059492 A2	12-10-2000